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13. ABSTRACT *(Maximum 200 words)*

This project has as its basis the discovery in my laboratory of a unique drug-responsive and growth-related hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity at the external cell surface. In metastasis models, tNOX inhibitors have slowed or prevented the invasive spread of cancer. The present project was to develop one or more lead drugs to reduce metastases in mice bearing transplanted mammary carcinomas. Glaucarubolone, a natural product tNOX inhibitor, both free and conjugated to polyethylene-glycol 5000, was prepared in quantities sufficient for animal testing. Free glaucarubolone supplied intra-tumorally was toxic and ineffective in the prevention of the formation of lung metastases. The conjugate was not toxic and a response was seen at the highest concentration tested of 5 mg/injection/mouse. In a collagen invasion assay, the glaucarubolone-PEG conjugate was effective at concentrations between 10^{-8} and 10^{-10} M. Green tea catechins were explored as potentially clinically useful redox potentiators of glaucarubolone anticancer activity. A relatively nontoxic mixture of glaucarubolone, epicatechin and epigallocatechin gallate was found to have excellent activity against 4T1 mouse mammary cancer cells and will be explored further as a new tNOX-based therapeutic strategy for management of breast cancer patients.

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TABLE OF CONTENTS

(5) INTRODUCTION

My laboratory described a 34 kD cell surface protein with NADH oxidase and protein disulfide-thiol interchange activity involved in the metastatic process. A small cadre of antitumor agents (sulfonylureas, vanilloids and quassinoids) that bind to the protein, presumably via a common quinone-binding site, emerge as potentially effective and relatively non-toxic anti-metastatic agents. The drug binding site is at the exterior of the cell such that drugs need not enter cells to be effective. Therefore, the inhibitors can be immobilized to impermeant supports to further increase selectivity and specificity. Studies of inhibitors, free and conjugated, of the drug-responsive NADH oxidase protein conducted with the B-16 mouse melanoma and other rodent cells lines were extended to a metastatic mouse mammary carcinoma. The anticipated long-range outcome is a drug-based strategy of metastatic prevention with the potential to increase substantially survival of breast cancer patients with regional lymph node involvement (Stage II) as well as other disease stages where distant metastases are not yet fully developed (Stage III).

(6) BODY

Task 1. Preparation and biological activity testing of glaucarubolone amino polyethylene glycol (PEG) conjugate.

Glaucarubolone (500 mg) was prepared for the project by Dr. Paul A. Grieco, Department of Chemistry and Biochemistry, Montana State University, Bozeman, Montana by extraction from the root bark of the desert shrub *Castela polyandra*. Another 500 mg of derivatized glaucarubolone was prepared for linking the amino polyethyleneglycol to form the glaucarubolone-polyethyleneglycol (glaucarubolone-PEG) conjugate.

For stereospecific conjugation via the $C_{(15)}$ hydroxyl group, we prepared II (Appendix Fig. 1) using a procedure that differentiated the $C_{(1)}$, $C_{(12)}$ and $C_{(15)}$ hydroxyl groups in glaucarubolone. Exposure of glaucarubolone to TMSOTf in pyridine containing triethylamine (0°C, RT, 1 h) gave rise (75% yield) to tris-trimethylsilylated material III (Appendix Fig. 1) in which the $C_{(12)}$ hydroxyl was exposed. Exposure of III to tetrabutylammonium fluoride in THF cleaved exclusively in excellent yield the $C_{(15)}$ OTMS ether. Treatment of IV (Appendix Fig. 1) with succinic anhydride in methylene chloride containing trimethylamine and 4-dimethylaminopyridine gave rise (80%) after treatment with HF/CH_3CN to II (Appendix Fig. 1) which was then conjugated with amino polyethyleneglycol. Note that the $C_{(12)}$ hydroxyl was sufficiently hindered to allow stereospecific acylation of the $C_{(15)}$ hydroxyl giving rise exclusively to II (Appendix Fig. 1). The derivatized glaucarubolone (II, Fig. 1) was coupled to amino polyethyleneglycol (Ave MW 5,000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

Task 2. Evaluation of anti-metastatic activity of glaucarubolone-amino PEG conjugate against metastatic breast cancer in mouse model.

A problem with glaucarubolone as a cancer therapeutic agent has been its rather low margin of safety. The EC_{50} for free glaucarubolone for the human mammary epithelial cell line MCF10A is only one order of magnitude greater than that for the human mammary carcinoma cell line BT-20. The murine 4T1 breast cancer cell line is as resistant to free glaucarubolone as is the mammary epithelia cell line MCF10A. However, when tested with the glaucarubolone-PEG conjugate, the EC_{50} is shifted by about 1 order of magnitude to lower drug concentrations (Manuscript I).

With free glaucarubolone, the two highest doses of 5 and 10 mg/animal/injection were toxic. Even at the near maximum tolerated dose of 2.5 mg/animal/injection, no tumor response was seen either from the standpoint of inhibition of growth of the primary tumor of inhibition of metastases to the lungs of the animals bearing tumors (Table 7 of Manuscript I).

In contrast to free glaucarubolone, the conjugate of glaucarubolone with PEG was not toxic at 5 mg/animal/injection (Table 8 of Manuscript I). At this concentration, the glaucarubolone-PEG conjugate was cytostatic to the growth of the murine mammary carcinoma cells in the mice (Fig. 3 of Manuscript I) whereas at 2.5 mg/animal/injection, the tumors grew to approximately the same size as controls.

Despite the cytostatic observations with the glaucarubolone-PEG conjugate at day 5 following the beginning of treatment, there was no significant slowing of tumor growth or of metastasis at the end of the experiment (Table 8 of Manuscript I). Higher concentrations of the glaucarubolone-PEG conjugate could not be tested due to problems of solubility whereas toxicity was dose limiting to glaucarubolone. The latter problem was overcome by combining the glaucarubolone with a mixture of catechins (Manuscripts II and III).

Task 3. Evaluation of activity of anti-metastatic vanilloids in the mouse metastatic breast cancer model.

Both the vanilloid vanillylamine and the quassinoid glaucarubolone were tested in the BALB/c mice carrying subcutaneous experimental tumors derived from the 4T1 cell line. The dose limiting toxicity of vanillylamine had been determined previously.

Vanillylamine given at a dose of 1 mg/mouse per injection beginning on alternate days after palpable tumor masses were discernable for a total of 6 injections was without effect either on tumor growth or lung metastases (Table 6 of Manuscript I). However, when combined with 0.2 mg ascorbate/ animal/injection, a reduction both of tumor mass and numbers of metastases was observed. Ascorbate alone at 0.2 mg/animal/injection was without effect. For vanillylamine to be active in restricting tumor growth and metastases, the target protein must be oxidized. This is effected in the experimental system with either ascorbate (Table 6 of Manuscript I) or t-butylhydroperoxide (e.g., Table 3 of Manuscript I).

Task 4. Growth studies in cells and mechanistic studies.

A series of specific tests were carried out to evaluate the effect of both the quassinoid glaucarubolone, its conjugate with polyethyleneglycol (PEG) and the vanilloid, vanillylamine on different metastatic parameters in laboratory models. These studies were carried out in collaboration with the laboratory of Prof. Marc Mareel, University Hospital, Ghent, Belgium. To measure the epithelial Ca^{2+}-dependent cell-cell adhesion mediated by E-cadherin when linked to the actin cytoskeleton via various catenins, the slow aggregation assay was carried out (Table 1 of Manuscript I) comparing two human breast cancer cell lines (MCF-7/AZ and MCF-7/6) and a human colonic carcinoma (HCT-8/R1). At the concentrations tested, none of the drugs had any effect on cell aggregation such that inhibitory effects were not directed to involvement of the E-cadherine-α-catenin system.

Collagen invasion assays, in contrast, did reveal activity (Tables 2 and 3 of Manuscript I). Here, type I collagen was mixed with culture medium to achieve a final concentration of 0.09% collagen. The ability of cells to invade into the collagen was estimated microscopically after 24 and 48 h. Results are expressed as percent of cells invading. The DHB-FIB is an invasive cell line whereas the MCF-7/AZ is a non-invasive breast cancer cell line.

In the first experimental series with the collagen invasion assay (Table 2 of Manuscript I), invasion with the invasive DHD-FIB cell line was reduced from 8.4% to 2.0% by a 10^{-8} M concentration of the glaucarubolone-PEG conjugate. The conjugate was at least twice as effective as free glaucarubolone. Capsaicin at 1 μM also inhibited in the collagen invasion assay.

In the second experimental series with the collagen invasion assay (Table 3 of Manuscript I), the glaucarubolone-PEG conjugate was most effective at a concentration of 10^{-10} M and equivalent at that concentration to 1 μM capsaicin and more effective than 0.01 to 1 μM of the free glaucarubolone. The concentration dependence in this assay was unusual and similar anomalies were observed in experiments with isolated plasma membrane vesicles in the inhibition of NADH oxidase (Fig. 2 of Manuscript I).

In the chick heart invasion assay (Table 4 of Manuscript I), cells were confronted with fragments of embryonic chick heart and invasion was scored on serial histological sections. With the PHF/MO4 cells on day 4, there was no effect of any of the compounds tested on invasion of the chick heart fragments. However, with the PHF/Bowes Melanoma on day 4 there was a response to the glaucarubolone-PEG conjugate not given by free glaucarubolone (Table 4 of Manuscript I).

Task 5. Characterization of tNOX target from metastatic mouse mammary tumor cells in culture and in situ.

With the antitumor sulfonylurea Ly181984 and with capsaicin, the activity both in terms of inhibition of HeLa cell growth and in inhibition of the plasma membrane NADH oxidase was very much dependent upon redox state (Manuscript III). For the antitumor sulfonylureas, activity was enhanced under reducing conditions whereas, for capsaicin, oxidizing conditions were required for maximum activity. Similarly, results were obtained with the 4T1 mammary cell line. With glaucarubolone, the inhibition was favored under oxidizing conditions especially for the conjugate where the EC_{50} for activity was lowered substantially in the presence of 100 μM oxidized glutathione compared to reduced glutathione. The enhanced activity in the presence of oxidized glutathione also was seen as an enhanced inhibition of 4T1 cell growth especially by the glaucarubolone conjugate under the oxidizing conditions afforded by GSSG. With capsaicin, the inhibition of growth of human and mouse melanoma cells *in vitro* and of mouse melanoma *in vivo* also was dependent upon oxidizing conditions. These findings suggest the possibility of therapeutic benefit both from immobilization of glaucarubolone to restrict its action to cell surface sites and from modification of the redox environment by means of co-administration to the tumor site of glaucarubolone or glaucarubolone-conjugates and mild oxidizing agents such as oxidized glutathione or *tert*-butylhydroperoxide.

A search for suitable modifiers of the redox environment to enhance the action of either glaucarubolone, the glaucarubolone conjugate with PEG or both lead to identification of tea catechins as also being targeted in their anticancer action to the tNOX glaucarubolone target (Manuscript II). When combined with glaucarubolone (or the glaucarubolone-PEG conjugate) inhibition of NOX activity was enhanced by one order of magnitude and cell killing was greatly enhanced (Manuscript III). The mixtures of glaucarubolone plus tea catechins were well tolerated by mice and appear to have exceptional promise as therapeutics for breast cancer management (See Task 6).

Task 6. Data analyses, preparation of findings for publication and planning for next level of implementation.

The anticancer quassinoid, glaucarubolone, inhibits both growth and a plasma membrane NADH oxidase associated with the surface of cancer cells. The inhibition was enhanced by tea catechins with a crude mixture of tea catechins more effective than the principal anticancer catechin, epigallocatechin gallate (EGCg). A 3-way mixture of epicatechin (EC), EGCg and glaucarubolone was more effective in inhibiting the cancer-associated NADH oxidase and growth of both HeLa and 4T1 mouse metastatic breast cancer cells than were individual components of the mixtures or individual components added two at a time. Neither EGCg nor EC affects the growth of nontransformed cell lines in culture (human mammary epithelia MCF-10A). The results offer an opportunity to utilize relatively safe and non-toxic tea catechins to enhance the response of tumor cells to an anticancer drug targeted to the plasma membrane NADH oxidase of the cell surface.

Preliminary animal studies are encouraging and plans have been formalized to continue the therapeutic investigations in collaboration with Pharmanex, Inc., Brisbane, CA. One publication and 3 manuscripts have resulted from the work. One continuation grant (Pharmanex) has been approved and funded and a second, a SBIR, has been submitted in collaboration with Bioanalytical Systems, Inc. of Lafayette, Indiana to develop analytical methods required to establish dosing regimens and for planned pre-clinical studies.

(7) KEY RESEARCH ACCOMPLISHMENTS:

- Redox dependence of quassinoid efficacy in NOX inhibition
- Search for therapeutic weak oxidants
- Identification of green tea catechins as redox-independent tNOX inhibitors
- Quassinoid-green tea catechin combinations for breast cancer therapy

(8) REPORTABLE OUTCOMES

Publications:

Morré, D. J., P. A. Grieco and D. M. Morré. 1998. Mode of action of the anticancer quassinoids. Inhibition of the plasma membrane NADH oxidase. Life Sciences 63, 595-604.

Manuscripts:

Morré, D. J., S. Caldwell, D. M. Morré, M. Mareel and E. A. Bruyneel. Antimetastatic activity of glaucarubolone, an anticancer quassinoid.

Morré, D. J., A. Bridge, L.-Y. Wu and D. M. Morré. Epigallocatechin gallate inhibits preferentially the NADH oxidase and growth of transformed cells in culture.

Morré, D. J. and D. M. Morré. Activity of the anticancer quassinoid, glaucarubolone, enhanced by tea catechins.

Funding received:

tNOX – A tea catechin and coenzyme Q target. Pharmanex, Inc. 4/1/99-3/31/00, $158,206.

Funding applied for:

Serum Analysis of Anticancer Tea Catechins, NIH SBIR, 3/1/00-2/28/01, $100,000 (Phase I).

(9) CONCLUSIONS

A cancer-specific cell surface hydroquinone (NADH) oxidase with protein disulfide thiol interchange activity (tNOX) was identified as a target for chemical intervention in breast cancer. Anticancer quassinoids, either free or conjugated, among the most potent tNOX inhibitors, were tested as inhibitors of metastatic breast cancer using a mouse model and with cells in culture. A potential limitation to the therapeutic efficacy of the quassinoids was identified as a dependency on the redox environment of the tNOX target. Weak oxidizing agents (dilute hydrogen peroxide, *tert*-butylhydroperoxide and N-chlorosuccinamide) were explored and found to be clinically impractical. Green tea catechins and polyphenols in general were identified as potentially clinically useful redox potentiators of the anticancer quassinoids. Especially effective was the principal tea catechin, epigallocatechin gallate (EGCg), which was inhibitory in the nanomolar range. The NADH oxidase and growth of normal cells and tissues were not inhibited by either the quassinoid or the catechin at therapeutic dose levels either alone or in combination. With cancer cells, enlargement and further growth was blocked. The cells eventually underwent programmed cell death (apoptosis). The tNOX target is on the external cell surface so that drugs need not enter cells to be effective and may be immobilized to decrease toxicology and increase efficacy. As such the findings identify a new tNOX-based therapeutic strategy with potential utility in the management of breast cancer patients.

(10) References:

Morré, D. J., P. A. Grieco and D. M. Morré. 1998. Mode of action of the anticancer quassinoids. Inhibition of the plasma membrane NADH oxidase. Life Sciences 63, 595-604.

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Manuscript I

ANTIMETASTATIC ACTIVITY OF GLAUCARUBOLONE, AN ANTICANCER QUASSINOID

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Summary

In previous work, a plasma membrane-associated hydroquinone (NADH) oxidase interchange activity with protein disulfide-thiol (designated tNOX) of transformed cells was shown to be inhibited by nanomolar and subnanomolar concentrations of the antitumor quassinoid, glaucarubolone. In a B-16 melanoma metastasis model, tNOX inhibitors did prevent the invasive spread of cancer. The present report was to determine the efficacy of both free glaucarubolone and a glaucarubolone conjugated to polyethylene-glycol 5000 to prevent metastases in a mouse breast cancer model. The EC_{50} for the glaucarubolone on the growth of 4T1 mouse mammary carcinoma cells was determined to be > 1 µM. That for the conjugate was < 1 µM. In a collagen invasion assay, the glaucarubolone-PEG conjugate was effective at concentrations between 10^{-8} and 10^{-10} M. Glaucarubolone efficacy in inhibition tNOX was enhanced under oxidizing conditions. Free glaucarubolone supplied intra-tumorally to mice bearing transplanted mammary carcinomas was toxic and ineffective in the prevention of the formation of lung metastases. The conjugate was not toxic and a response was seen at the highest concentration tested of 5 mg/injection/mouse.

Key Words: quassinoids, antitumor agents, metastasis, growth inhibition, glaucarubolone, NADH oxidase, plasma membranes, HeLa cells, liver

Introduction

Our laboratory has discovered and characterized a 34 kD protein associated with the cell surface of cancer cells (1). The protein exhibits protein disulfide-thiol interchange (2) and hydroquinone (NADH) oxidase (NOX) (3) activities that are inhibited by a small cadre of quinone site inhibitors (1). The site is at the external cell surface (4) and drugs need not enter cells to be effective (5, 6). Using a B-16 mouse melanoma model, we have demonstrated that inhibition of the NOX activities of the melanoma cell plasma membrane results in blockage of the metastatic spread of the cancer (7). As metastatic spread is the principal cause of treatment failure in breast cancer, this study was to extend these findings to metastatic breast disease. A quassinoid, glaucarubolone, having demonstrated activity against breast cancer, was immobilized to polyethylene glycol (PEG), an impermeant support to increase its margin of safety and to be tested as an anti-metastatic agent of potentially high specificity and selectivity for use in breast cancer management.

The growth factor- and hormone-responsive protein disulfide-thiol interchange protein of the plasma membrane with NADH oxidase activity (8, 9) appears to be involved functionally in physical membrane displacements related to cell enlargement and cancer metastasis (8). In transformed cells, the activity is constitutively activated and no longer hormone- and growth factor-responsive (10, 11). Additionally, the cancer form of the activity (designated tNOX) is specifically inhibited by thiol reagents (12) and by a small cadre of antitumor drugs whose sites of action, until now, have remained elusive. These drugs include the antitumor sulfonylureas (13), certain vanilloids such as capsaicin (8-methyl-N-vanillyl-6-noneamide) (11) and the anticancer quassinoid, glaucarubolone (14). Drugs of all three groups appear to occupy a quinone site on the protein, a site that also can be occupied in a functional manner by the natural quinone cofactor, ubiquinone (coenzyme Q_9) (15). The protein is anchored via its C-terminus at the outer surface of the plasma membrane (4). Both protein and drug site are external and drugs directed to the responsive cell surface NADH oxidase need not enter the cells to be effective (4-6).

HeLa and other cancer cells inhibited by drugs targeted to the responsive NADH oxidase fail to enlarge normally following cytokinesis (16). Cells must reach a minimal size in order to re-enter mitosis (17). The small cells resulting from treatment with antitumor quassinoids, vanilloids or sulfonylureas, subsequently fail to enter mitosis and undergo apoptotic cell death.

Preliminary studies were with human mammary adenocarcinoma (11) and other human cell lines in culture (7). Using the B-16 mouse melanoma carried in C57BL/6 mice, experiments were extended to animals for the vanilloids (7). Tumor growth was effectively reduced but an unexpected outcome of these studies was the effectiveness of the vanilloids to reduce metastatic spread of the B-16 melanoma line.

Also effective as a potent NOX inhibitor was the antitumor quassinoid, glaucarubolone (14). Glaucarubolone is a representative of a series of both naturally occurring and chemically-modified plant products from the family Simbouriaceae with potent anti-cancer activity (18). These compounds have been tested extensively on M17 Adr mouse mammary carcinoma cells. Especially effective was the hydroxy analog of glaucarubolone which demonstrated solid tumor selectivity to both murine and human cells but also selectivity to M17/adr. Additional specificity to glaucarubolone has been imparted by conjugates involving the C-15 hydroxyl. Effectiveness of a C-15 acylated analog ($R=COCH_2NMe_2$) of glaucarubolone against MAM 16/C/RP cells) (% T/C of 16) has been demonstrated. A 2% T/C equal to < 10 constitutes a highly active agent and would represent $>$ 1 log cell kill. Since the drug-responsive site of the NADH oxidase is located at the cell's exterior, inhibitory drugs directed to the site need not enter cells to be effective (4-6). Impermeant conjugates, therefore can be used an antitumor agents. These conjugates also help overcome a potential drawback of the quassinoid antitumor agents, that of a relatively high inherent cytotoxicity and narrow therapeutic ratio. By reducing or eliminating unspecific cytotoxicity and restricting the drug to the cell surface target, conjugation of quassinoids to impermeant supports greatly increases efficacy and reduces toxicity with a corresponding 10- to 100-fold potential increase in the margin of safety.

One such conjugate where preliminary data are available (14) is a conjugate of glaucarubolone through the C-15 hydroxyl to amino polyethylene glycol (PEG).

The amino-PEG-glaucarubolone is more effective than free glaucarubolone in inhibiting the target NADH oxidase of plasma membrane, exhibits enhanced water solubility compared to glaucarubolone and is more effective than glaucarubolone in inhibiting the growth of HeLa and 4T1 mouse mammary cancer cells.

Materials and Methods

Female BALB/c mice 8 to 12 weeks old were obtained from a commercial supplier (Harlan Industries, Indianapolis, IN) and housed in the Purdue University Small Animal Facility following good animal practice procedures adopted by the University-wide animal care committee.

Tumor cell lines. Tumor subpopulation line 4T1 arising from a BALB/cf C3H mouse were utilized (19, 20). The cells were grown in DME-10, Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). To prepare cells for injection, flasks were rinsed with 0.25% trypsin in 0.5% EDTA and washed once, and suspended in DME-10.

Experimental metastasis. Cells from monolayer culture were suspended in Hank's buffered salt solution and 1 x 10^5 cells were injected s.c. into mice in the subscapular region in a volume of 0.1 ml. Primary tumors were measured twice a week in 2 perpendicular dimensions using a vernier caliper. Tumor mass in cm^3 was calculated by the formula a x $b^2/2$, where b is the smaller and a is the larger of the two dimensions. To estimate doubling time, the values were fitted to an exponential growth curve using linear regression analysis of the logarithm of tumor volume.

Anti-metastatic drugs were administered intratumoral on alternate days beginning after palpable tumor masses were discernible. Appropriate solvent and sham-injected controls were included. At 15 days post tumor implantation mice were sacrificed and major organs (e.g. lung, liver, lymph nodes) were examined for evidence of metastases.

Invasion in vitro. Embryonic chick heart fragments were incubated with adenocarcinoma cells (21). After 4 days of confronting culture in liquid medium, the heart fragments were fixed in Bouin solution, processed for paraffin embedding and sectioned serially. Consecutive sections, stained with hematoxylin-eosin were used for histological evaluation of the interaction between the heart fragments and adenocarcinoma cells. The extent of interactions were assigned a numerical rating as described (22). To examine the effect of potential antimetastatic agents on the interaction between adenocarcinoma cells and the heart fragments, the agents were dissolved in the medium during the preculture period (4 days) of the heart fragments, during the confronting culture period or during both periods. Concentrations ranged between 0.000 and 0.100 mM. Reversibility of a possible anti-invasive effect of the agents was tested by changing the medium after 4 days of confronting culture for agent-free medium and further culturing during another 4 days.

Viability of aggregates and heart fragments. Individual heart fragments and adenocarcinoma aggregates were treated with different concentrations of potential antimetastatic agents for 8 days and 4 days respectively, under similar circumstances as for confronting cultures. The aggregates were then explanted in agent-free culture medium on a tissue culture plastic substrate as described (22). The number of explants showing radial outgrowth on the substrate versus the total number of explants were considered as a measure of cytotoxicity.

Growth of cell aggregates. Spheroidal adenocarcinoma aggregates with an initial diameter of 0.2 mm were grown in suspension culture for 7 days in the presence or absence of the antimetastatic agents under test. Changes in volume were calculated from daily measurements of aggregate diameters using an inverted microscope fitted with an ocular micrometer.

Colony formation. Approximately 1 ml portions of solid agar medium containing 1.5×10^4 adenocarcinoma cells in suspension were incubated in 35 mm diameter plastic Petri dishes at 37°C for 7 days. Culture dishes were then placed individually on a cross-hatched plastic matrix. Groups of cells (> 3 cells) were counted and colony diameters measured.

Morphology and motility of individual adenocarcinoma cells. Observations were made on HeLa cells in 25 cm^2 plastic tissue culture flasks in medium with or without potential antimetastatic agents. For observation and image analysis, video films were made using a Wild inverted microscope with phase contrast optics (10 x objective) in a thermostatic chamber (37 ± 0.5°C), a National Panasonic WV-1850C video camera and a Sony Umatic VO-585P video recorder equipped with an AC-580 animation control unit and a time-date generator. Analysis of morphology was carried out on real-time video films, and analysis of motility was on time-lapse video films using a Bueckler microscaler.

Isolation of mouse mammary plasma membrane. Procedures followed those developed in our laboratory for isolation of plasma membranes from human mammary adenocarcinoma and other cultured cell lines using aqueous two-phase partition (20). The purity of the plasma membrane was determined by electron microscope morphometry and assay of marker enzymes.

Spectrophotometric measurement of NADH oxidase. NADH oxidase activity was determined at 37°C as the disappearance of NADH measured at 340 nm. Activity was measured using a Hitachi U3210 with stirring and continuous recording over 5 min intervals. The reaction mixture contained 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN and 150 μM NADH. A millimolar extinction coefficient of 6.22 was used to calculate the rate of NADH disappearance.

Spectrophotometric measurement of protein disulfide-thiol interchange activity. Protein disulfide-thiol interchange interchange activity was monitored spectrophotometrically at 340 nm by following the cleavage of 2,2′ dithiopyridine.

Glaucarubolone-PEG conjugation. Glaucarubolone (1) isolated from the roots of *Castela polyandra* was provided by Dr. Paul Grieco, Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT (see below). A C(15) succinylated derivative of glaucarubolone 2 was coupled to amino polyethyleneglycol (ave MW 5000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

For stereospecific conjugation via the $C_{(15)}$ hydroxyl group, we prepared II (Fig. 1) using a procedure that differentiated the $C_{(1)}$, $C_{(12)}$ and $C_{(15)}$ hydroxyl groups in glaucarubolone. Exposure of glaucarubolone to TMSOTf in pyridine containing triethylamine (0° C, RT, 1 h) gave rise (75% yield) to tris-trimethylsilylated material III (Fig. 1) in which the $C_{(12)}$ hydroxyl was exposed. Exposure of III to tetrabutylammonium fluoride in THF cleaved exclusively in excellent yield the $C_{(15)}$ OTMS ether. Treatment of IV (Fig. 1) with succinic anhydride in methylene chloride containing trimethylamine and 4-dimethylaminopyridine gave rise (80%) after treatment with HF/CH_3CN to II (Fig. 1) which was then conjugated with amino polyethyleneglycol. Note that the $C_{(12)}$ hydroxyl was sufficiently hindered to allow stereospecific acylation of the $C_{(15)}$ hydroxyl giving rise exclusively to II (Fig. 1). The derivatized glaucarubolone (II, Fig. 1) was coupled to amino polyethyleneglycol (Ave MW 5,000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

Results

A series of specific tests were carried out to evaluate the effect of both the quassinoid glaucarubolone, its conjugate with polyethyleneglycol (PEG) and the vanilloid, vanillylamine on different metastatic parameters in laboratory models. To measure the epithelial Ca^{2+}-dependent cell-cell adhesion mediated by E-cadherin when linked to the actin cytoskeleton via various catenins, the slow aggregation assay was carried out (Table 1) comparing two human breast cancer cell lines (MCF-7/AZ and MCF-7/6) and a human colonic carcinoma (HCT-8/R1). At the concentrations tested, none of the drugs had any effect on cell aggregation such that inhibitory effects were not directed to involvement of the E-cadherin-γ-catenin system.

Collagen invasion assays, in contrast, did reveal activity (Tables 2 and 3). Here, type I collagen was mixed with culture medium to achieve a final concentration of 0.09% collagen. The ability of cells to invade into the collagen was estimated microscopically after 24 and 48 h. Results are expressed as percent of cells invading. The DHB-FIB is an invasive cell line whereas the MCF-7/AZ is a non-invasive breast cancer cell line.

In the first experimental series with the collagen invasion assay (Table 2), invasion with the invasive DHD-FIB cell line was reduced from 8.4% to 2.0% by a 10^{-8} M concentration of the glaucarubolone-PEG conjugate. The conjugate was at least twice as effective as free glaucarubolone. Capsaicin at 1 μM also inhibited in the collagen invasion assay.

In the second experimental series with the collagen invasion assay (Table 3), the glaucarubolone-PEG conjugate was most effective at a concentration of 10^{-10} M and equivalent at that concentration to 1 μM capsaicin and more effective than 0.01 to 1 μM of the free glaucarubolone. The concentration dependence in this assay was unusual and similar anomalies were observed in experiments with isolated plasma membrane vesicles in the inhibition of NADH oxidase (Fig. 2).

In the chick heart invasion assay (Table 4), cells were confronted with fragments of embryonic chick heart and invasion was scored on serial histological sections. With the PHF/MO4 cells on day 4, there was no effect of any of the compounds tested on invasion of the chick heart fragments. However, with the PHF/Bowes Melanoma on day 4 there was a response to the glaucarubolone-PEG conjugate not given by free glaucarubolone (Table 4).

As a cancer therapeutic agent free glaucarubolone exhibits a rather low margin of safety. This is illustrated in Table 5 where the EC_{50} for free glaucarubolone for the human mammary epithelial cell line MCF10A is only one order of magnitude greater than that for the human mammary carcinoma cell line BT-20. The murine 4T1 breast cancer cell line is as resistant to free glaucarubolone as is the mammary epithelia cell line MCF10A. However, when tested with the glaucarubolone-PEG conjugate, the EC_{50} is shifted by about 1 order of magnitude to lower drug concentrations.

Both the vanilloid vanillylamine and the quassinoid glaucarubolone were tested in the BALB/c mice carrying subcutaneous experimental tumors derived from the 4T1 cell line. The dose limiting toxicity of vanillylamine had been determined previously.

Vanillylamine given at a dose of 1 mg/mouse per injection beginning on alternate days after palpable tumor masses were discernable for a total of 6 injections was without effect either on tumor growth or lung metastases (Table 6). However, when combined with 0.2 mg ascorbate/animal/injection, a reduction both of tumor mass and numbers of metastases was observed. Ascorbate alone at 0.2 mg/animal/injection was without effect. For vanillylamine to be active in restricting tumor growth and metastases, the target protein must be oxidized. This is effected in the experimental system with either ascorbate (Table 6) or t-butylhydroperoxide (e.g., Table 3).

With free glaucarubolone, the two highest doses of 5 and 10 mg/animal/injection were toxic. Even at the near maximum tolerated dose of 2.5 mg/animal/injection, no tumor response was seen either from the standpoint of inhibition of growth of the primary tumor of inhibition of metastases to the lungs of the animals bearing tumors (Table 7).

In contrast to free glaucarubolone, the conjugate of glaucarubolone with PEG was not toxic at 5 mg/animal/injection (Table 8). At this concentration, the glaucarubolone-PEG conjugate was cytostatic to the growth of the murine mammary carcinoma cells in the mice (Fig. 3) whereas at 2.5 mg/animal/injection, the tumors grew to approximately the same size as controls.

Despite the cytostatic observations with the glaucarubolone-PEG conjugate at day 5 following the beginning of treatment, there was no significant slowing of tumor growth or of metastasis at the end of the experiment (Table 8). Higher concentrations of the glaucarubolone-PEG conjugate could not be tested due to problems of solubility.

Discussion

Both the quassinoid glaucarubolone and the vanilloids vanillylamine and capsaicin, inhibitors of the cell-surface, unregulated and drug-responsive NADH oxidase of cancer cells (tNOX) show antimetastatic activity in breast cancer models. Use of both capsaicin and free glaucarubolone in animal experiments is compromised by high toxicity and a low therapeutic ratio. However, with the conjugated glaucarubolone and with vanillylamine, dose-limiting toxicities have not been encountered. Drug conjugation presents a viable strategy to reduce toxicity without loss of antitumor activity with the tNOX inhibitors.

The basis for prevention of cell enlargement by tNOX inhibitors has been explained on the basis that the normal function of the enzymatic activity measured as an NADH oxidase may be to catalyze a protein disulfide-thiol interchange involved in physical membrane displacements important to growth and especially cell enlargement. In cancer, these reactions become unregulated and the unregulated activity becomes responsive to the small cadre of potential antitumor drugs (1) listed in the introduction. When inhibited, the cell surface displacement activities associated with growth and invasion of cancer cells are blocked (16).

Metastasis is a major factor in contributing to the incurability of cancer and the major determinant of the malignancy (24). Benign tumors remain confined to their regions or origin while local invasion marks the onset of malignancy.

Current concepts describe metastasis as a multistep process (25). Invasion from the primary cancer occurs mostly through the basement membrane into the surrounding tissues. This is followed by intravasation into lymph and blood. Following transport in the lymph or in the circulation extravasation leads to tumor formation at distant sites. Metastases of themselves are invasive tumors and may repeat the overall multistep process. By isolating each step of the process of metastatic process, it is possible to determine where in the cascade an antimetastatic drug may block.

Cell-cell adhesion molecules encoded by genes of the cadherin superfamily have been extensively studied with respect to influencing tissue organization (26-28). One of these cell-cell adhesion molecules, E-cadherin, is expressed in most epithelia and could contribute to the invasive phenotype if underexpressed or blocked in its action (29). When the epithelial Ca^{2+}-dependent cell-cell adhesion mediated by E cadherin in concert with various cateins was measured in the slow conjugation assay, the tNOX inhibitors tested were without effect.

Two *in vitro* systems were employed to examine effects on invasion. A Collagen I invasion assay determines the ability to cells to invade a collagen layer when ploted on the surface. In this assay the glaucarubolone-PEG conjugate consistently scored higher than free glaucarubolone.

A second assay system was the chicken heart invasion assay (21). In addition to the demonstration of an anti-invasive effect on PHF/Bowes melanoma invasion *in vitro*, the confronting cultures treated with glaucarubolone-PEG or conjugate gave some indications concerning the possible mechanisms of action of the quassinoid. First, treatment of the heart fragments during the preculture period or of the confronting cultures separately had no inhibitory effect on invasion. This indicates that the glaucarubolone target cannot be localized in the pre-cultured heart fragments exclusively. Second, its anti-invasive activity could not be ascribed to an irreversible cytotoxic effect on the melanoma cell population. This was confirmed by experiments showing that solitary melanoma aggregates were able to adhere to and migrate on tissue culture plastic after treatment with the glaucarubolone-PEG conjugate.

The effect on growth of melanoma cell aggregates was not a result of increased cell shedding from the aggregate into the liquid culture medium. Colony formation experiments showed that even when a soft agar medium prevented cell shedding, the glaucarubolone-PEG conjugate still inhibited growth of the cell groups. Growth inhibition, however, cannot be held responsible for the lack of invasion in confronting cultures treated with the glaucarubolone-PEG conjugate. One possible clue to explain the anti-invasive activity of the conjugate is the inhibition of cell motility. Further analysis of cell translocation indicated that the conjugate did not inhibit direction finding *per se*, but slowed both stationary and translocative cell motility on tissue culture plastic. A general inhibition of cell motility could well be invoked to explain the anti-invasive activity of the conjugate and this would be in accordance with the vast body of evidence showing that cell motility is necessary for invasion (30).

Few data in the literature are available concerning biological effects of glaucarubolone. This quassinoid, which appears to possess a potent antiviral activity as well, (31) inhibits very strongly the cancer associated cell surface NADH oxidase (tNOX).

In conclusion, both glaucarubolone and the glaucarubolone-PEG conjugate inhibit invasion of melanoma cells into embryonic chick heart *in vitro*, possibly by interfering with cell motility. Its mechanism of action is suggested to be via inhibition of the cell surface hydroquinone (NADH) oxidase with protein disulfide thiol interchange activity specific to the surface of cancer cells (tNOX) (1). The model consisted of aggregates of malignant tumor cells confronting chick heart fragments *in vitro* (21). The explanation is that locomotion of the confronting tumor cells is mediated through the same machinery as cell enlargement and that tNOX inhibitors prevent locomotion. As locomotion is considered to be necessary for invasion of tumor cells (32), any substance interfering with locomotion could stop or delay invasion.

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TABLE 1. Slow aggregation assay with MCF-7/6 cells.

Drug	MCF-7/AZ	MCF-7/6	HCT-8/R1
Untreated	+	±	-
Capsaicin 1 μM	+	±	-
Vanillylamine 10 μM + Ascorbic acid 10 μM	+	±	-
Glaucarubolone 10^{-8} M	+	±	-
Glaucarubolone-PEG conjugate 10^{-8} M	+	±	-

The MCF-7/6 cells formed aggregates with irregular borders. There was more compaction and larger aggregates than usual.

TABLE 2. Collagen invasion assays.

Cells	Treatment	Percent invasion
DHD-FIB	None	8.4
DHD-FIB	1 μM Capsaicin	5.7
DHD-FIB	10 μM Vanillylamine and 10 μM Ascorbate	7.4
DHD-FIB	10^{-8} Glaucarubolone	4.7
DHD-FIB	10^{-8} Glaucarubolone-PEG conjugate	2.0
MCF-7/AZ (noninvasive)	None	0.0

TABLE 3. Collagen invasion assays continued.

Cells	Treatment	Percent invasion
DHD-FIB	None	18.4
DHD-FIB	10^{-6} Glaucarubolone	8.3
DHD-FIB	10^{-7} Glaucarubolone	5.3
DHD-FIB	10^{-8} Glaucarubolone	5.2
DHD-FIB	10^{-8} Glaucarubolone-PEG conjugate	7.4
DHD-FIB	10^{-9} Glaucarubolone-PEG conjugate	3.8
DHD-FIB	10^{-10} Glaucarubolone-PEG conjugate	2.8
DHD-FIB	1 μM Capsaicin	2.9
DHD-FIB	10 μM Capsaicin + 10 μM t-Butylhydroperoxide	6.0
DHD-FIB	10 μM t-Butylhydroperoxide	3.7
MCF-7/AZ	None	0.0

TABLE 4. Chicken heart invasion assay.

	Grade of Invasion					
Confrontation	0	I	II	III$^-$	III	IV
PHF/MO4: d_4						3
+ Capsaicin						3
+ Vanillylamine + Ascorbic acid						3
+ Glaucarubolone-PEG conjugate						3
PHF/Bowes Melanoma: d_{a4}	1				2	
+ Capsaicin	1		1		1	
+ Vanillylamine + Ascorbic acid						3
+ Glaucarubolone						3
+ Glaucarubolone-PEG conjugate			2		1	

Concentrations as in Tables 1-3.

TABLE 5. EC_{50} in cell culture of glaucarubolone and the glaucarubolone-PEG conjugate.

Cell line	Glaucarubolone	Glaucarubolone-PEG conjugate
HeLa[1]	0.5 μM	1 μM
MCF10A[2]	1 μM	-
BT-20[3]	0.1 μM	-
4T1[4]	> 1 μM	< 1 μM

[1]Human cervical carcinoma

[2]Mammary epithelia (non-cancer)

[3]Human mammary adenocarcinoma

[4]Mouse mammary adenocarcinoma

TABLE 6. Effect of the vanilloid, vanillylamine, with and without ascorbic acid on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice.

Compound	Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	_	0.6 ± 0.2	2.8 ± 3
Vanillylamine	1 mg	0.64 ± 0.15	4 ± 1.6
Ascorbate	0.2 mg	0.6 ± 0.2	3.4 ± 1.5
Vanillylamine + Ascorbate	1 mg + 0.2 mg	0.48 ± 0.2	1.8 ± 1.3

Cells from monolayer culture were suspended in Hank's buffered salt solution and 1 x 10^6 cells were injected into 5 mice/treatment in the subscapular region in a volume of 0.1 ml. Primary tumors were measured twice a week in 2 perpendicular dimensions using a vernier caliper. Tumor mass in cm^3 was calculated by the formula a x $b^2/2$, where b is the smaller and a is the larger of the two dimensions.

Anti-metastatic drugs were administered in the dose/injection indicated intratumoral in 0.1 ml total volume on alternate days beginning after palpable tumor masses were discernible (approximately 7 days post implantation) for a total of 6 injections. Appropriate solvent and sham-injected controls were included. At 15 days post tumor implantation, mice were sacrificed and major organs (e.g., lung, liver, lymph nodes) were examined for evidence of metastases.

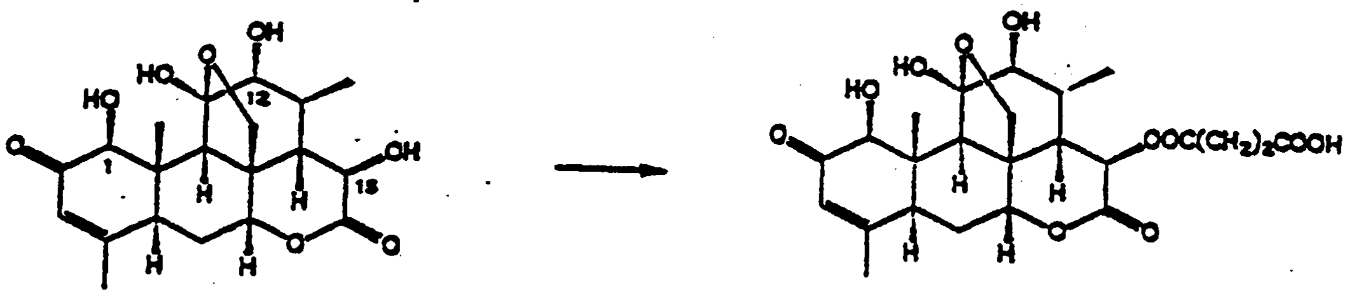
TABLE 7. Effect of glaucarubolone on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice. Experimental details as for Table 6.

Glaucarubolone, Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	1.1 ± 0.3	2 ± 2
1 mg	0.6[5]	11
2.5 mg	1.0 ± 0.5	4 ± 2
5 mg	Dead	
10 mg	Dead	

TABLE 8. Effect of glaucarubolone-PEG conjugate on tumor weight and metastases of 4T1 murine mammary carcinoma cells in BALBc mice. Experimental details as in Table 6.

Glaucarubolone-PEG conjugate Dose/injection/mouse	Tumor weight (g)	Lung metastases
None	3.3 ± 1.2	4 ± 0
1 mg	2.4	5
2.5 mg	2.9 ± 0.7	5 ± 2
5 mg	2.6	3

List of Figures



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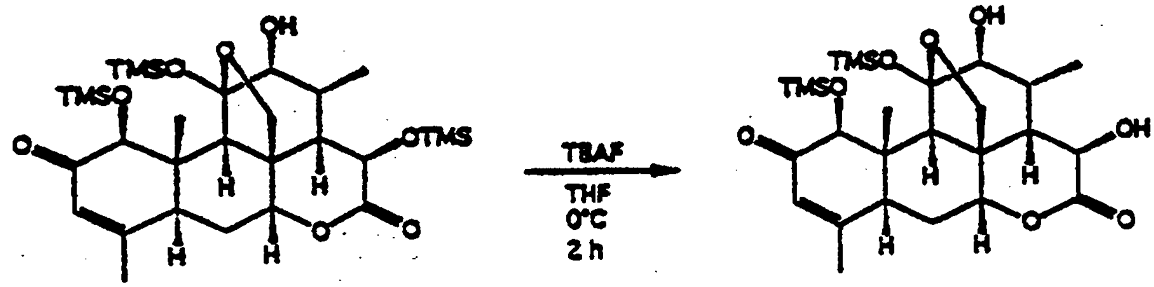

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Fig. 1

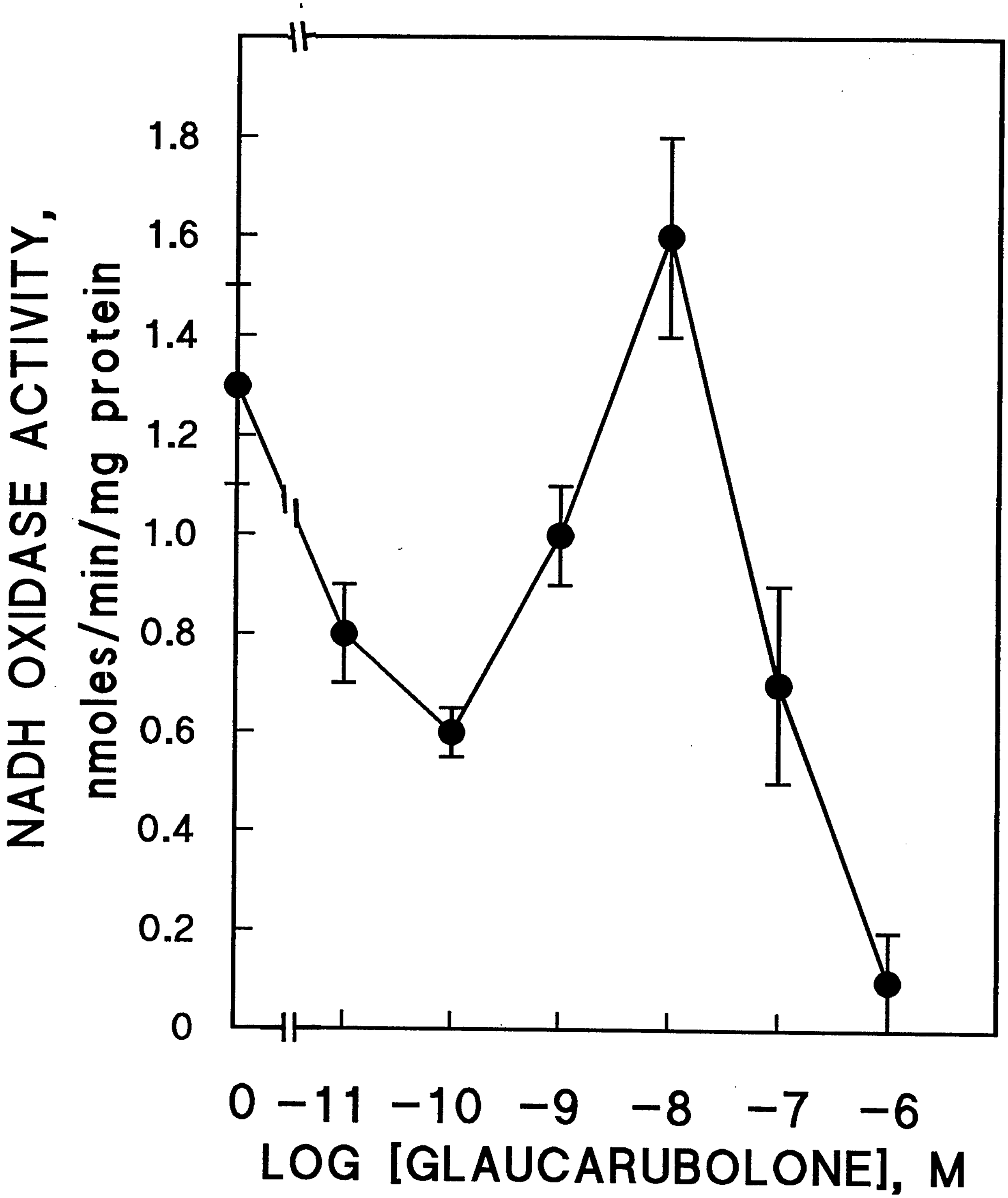
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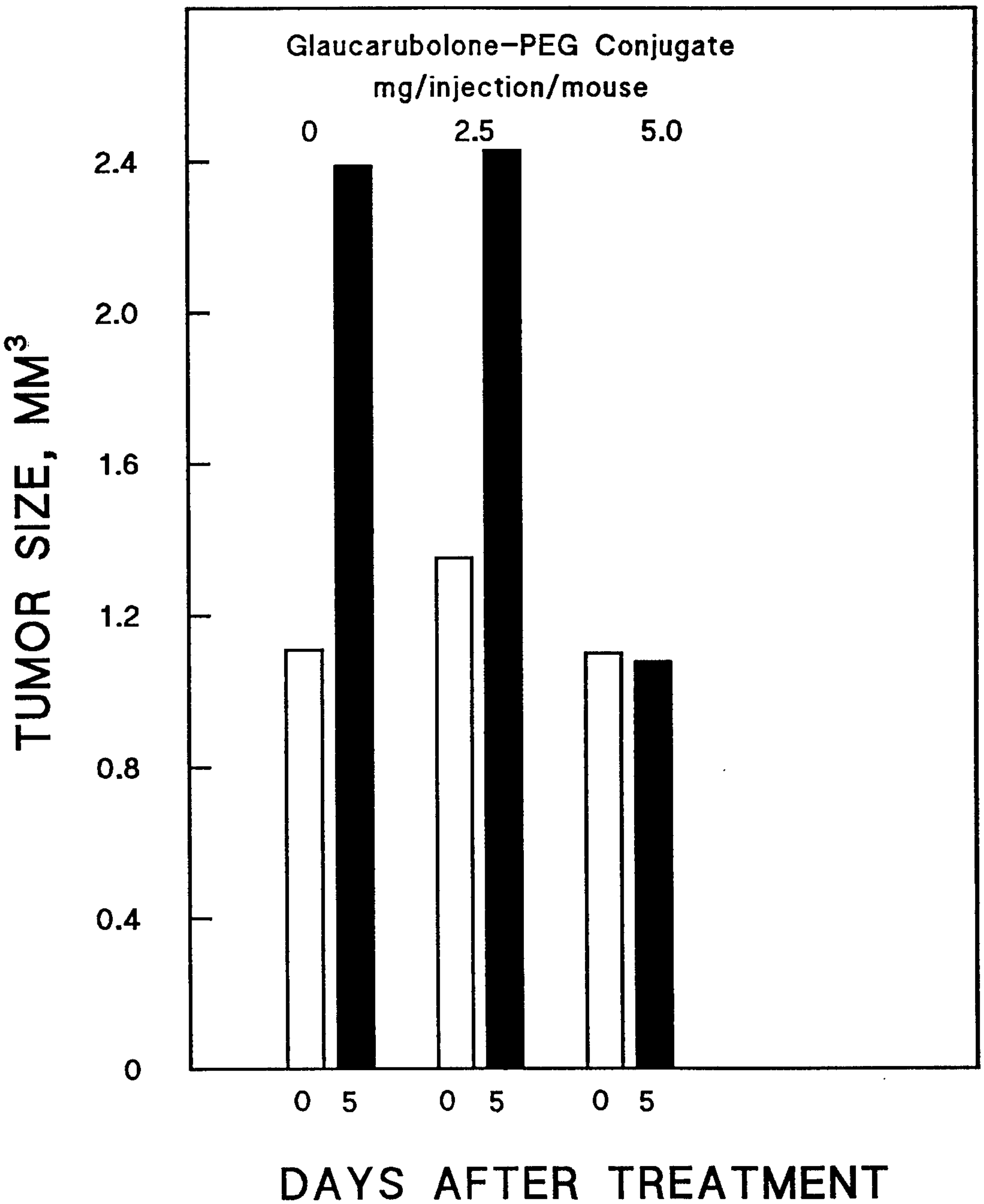
Fig. 2.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DAYS AFTER TREATMENT

Fig. 3

Manuscript II

EPIGALLOCATECHIN GALLATE INHIBITS PREFERENTIALLY THE NADH OXIDASE AND GROWTH OF TRANSFORMED CELLS IN CULTURE

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGCg, epigallocatechin gallate.

ABSTRACT

A drug-responsive and cancer-specific NADH oxidase of the mammalian plasma membrane, constitutively activated in transformed cells, was inhibited preferentially in HeLa and human and mouse mammary adenocarcinoma by the naturally-occurring catechin of green tea, epigallocatechin gallate (EGCg). With cells in culture, EGCg preferentially inhibited growth of HeLa and mammary adenocarcinomas compared to growth of mammary epithelial cells. Inhibited cells became smaller and cell death was accompanied by a condensed and fragmented appearance of the nuclear DNA as revealed by fluorescence microscopy with 4',6-diamidino-2-phenylindole (DAPI) suggestive of apoptosis. Mammary epithelial cells recovered from EGCg treatment even at 50 μM whereas growth of HeLa and mammary adenocarcinoma cells was inhibited by EGCg at concentrations as low as 1 μM with repeated twice daily doses and did not recover from treatment with 50 μM EGCg. The findings correlate inhibition of cell surface NADH oxidase activity and inhibition of growth with EGCg-induced apoptosis.

INTRODUCTION

Previous reports described a growth factor- and hormone-stimulated NADH oxidase activity of rat liver plasma membranes (1, 2). Several correlative studies have produced evidence for the involvement of this growth factor-responsive NADH oxidase in the control of cell proliferation (3). The activity in transformed cells and tissues was distinguished from that of liver in that the growth factor- and hormone-responsiveness was lost in plasma membranes of transformed liver tissues. These studies were with hyperplastic nodules of liver induced by the liver carcinogen, 2-acetylaminofluorene (4) and transplanted rat hepatomas (5).

The NADH oxidase activity of liver plasma membranes is unique among oxidoreductase activities not only in its response to growth factors and hormones but, also in its response to inhibitors and activators other than growth factors and hormones (3, 6, 7). To further characterize this unusual NADH oxidase activity, studies were extended to include responses to quinone analogs. The activity has been shown to require quinones (8) but is inhibited by the quinone analog capsaicin (8-methyl-N-vanillyl-6-noneamide) (9) and an antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) (10). The NADH oxidase activity of rat liver plasma membrane was largely unaffected by capsaicin and the antitumor sulfonylureas whereas the NADH oxidase activity of HeLa plasma membranes was strongly inhibited. The results suggested a fundamental difference in response to drugs between the NADH oxidase activities of normal and transformed cells and tissues that correlated with inhibition of growth and induction of apoptosis in the transformed cells. In the present report, we extend these observations to epigallocatechin gallate (EGCg), the putative active anticancer catechin polyphenol of green tea.

Focused research on the health benefits of tea is very recent (11). The major interest stems from high levels of antioxidant tea phenols including epigallocatechin gallate (EGCg), a predominant catechin polyphenol (12). Epidemiological studies show that cancer onset of patients in Japan who had consumed 10 cups of green tea per day was 8.7 years later among females and 3 years later among males, compared with patients who had consumed under three cups per day (13). As such, a possible relationship between high consumption of green tea and the low incidence of prostate and breast cancer in Asian countries where green tea consumption is high has been postulated (14, 15). Also reported has been preferential inhibition of growth and induction of apoptosis by EGCg in cancer cell lines compared to non-cancer cell lines (12, 16).

MATERIALS AND METHODS

Growth of Cells. HeLa (ATCC CCL2) cells were grown in 175 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/l gentamycin sulfate (Sigma). Cells were harvested by scraping and taken up in 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4 to a final cell concentration of 0.1 g wet weight (gww) per ml.

MCF-10A human mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's medium containing cholera enterotoxin (100 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (EGF, 20 mg/ml), and 5% horse serum. Media were renewed every 2-3 days.

BT-20 human breast adenocarcinoma cells were cultured in Eagle's minimal essential medium nonessential amino acids and Earle's balanced salts supplement with 10% fetal bovine serum. Media were renewed as for MCF-10A cells.

A mouse mammary tumor subpopulation line 4T1 arising from a BALB/cf C3H mouse (17, 18) was grown in DME-10, Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Cell lines were from the American Type Culture Collection (Rockville, MD).

Purification of Plasma Membranes from Cultured Cells. Cultured cells were collected by centrifugation for 6-15 min at 175-1000 X g. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10-30 min to swell the cells. Homogenization was achieved in 7- to 8-ml aliquots with a Polytron homogenizer (Brinkmann) for 30-40 sec at 10,500 rpm, using a PT-PA 3012/23 or ST-10 probe. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 175 X g to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4 x 10^6 g• min (e.g. 1 h at 23,500 X g) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of ≈ 1 ml per pellet from 5 x 10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis consisting of 6.6% (w/w) Dextran T-500 (Pharmacia) and 6.6% (w/w) Polyethylene Glycol 3350 (Fisher) in a 5 mM potassium phosphate buffer (pH 7.2) for aqueous two-phase separation as described (19, 20). The upper phase, enriched in plasma membranes, was diluted 5-fold with 1mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Preparation of HeLa cells and cell-free extracts. HeLa S cells were collected by centrifugation and shipped frozen in 0.1 M sodium acetate, pH 5 in a ratio of 1 ml packed cell volume to 1 ml of acetate (Cellex Biosciences, Minneapolis, MN). The cells were thawed at room temperature, resuspended and incubated at 37° C for 1 h to release the protein (21). The cells were removed by centrifugation at 37,000 g for 60 min and the cell-free supernatants were refrozen and stored in 1 ml aliquots at -70° C.

For heat treatment, 1 ml aliquots of the above supernatant material were thawed at room temperature and heated to 50° C for 10 min. The denatured proteins were removed by centrifugation (1,500 g, 5 min). Full activity was retained from this step (21).

For protease treatment, the pH of the heat-stable supernatant was adjusted to 7.8 by addition of 0.1 M sodium hydroxide. *Tritirachium album* proteinase K (Calbiochem) was added (4 ng/ml) and incubated at 37° C for 1 h with full retention of enzymatic activity and drug response (21). The reaction was stopped either by freezing for determination of enzymatic activity or by addition of 0.1 M phenylmethylsulfonyl fluoride (PMSF) in ethanol to yield a final concentration of 10 mM PMSF.

Spectrophotometric Assay of NADH Oxidase. NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 μM NADH at 37°C with stirring. Activity was measured using a Hitachi U3210 or SLM Aminco DW2000 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity. EGCg was added at the final concentrations indicated at the beginning of the assay and was present during the assay period.

Proteins were estimated by the bicinchonic acid method (22) with bovine serum albumin as standard.

Fluorescence Microscopy. Cells were grown for 72 h on glass coverslips placed in small culture dishes with media containing 100 μM EGCg in ethanol or an equivalent amount of ethanol alone. The coverslips were rinsed and the cells fixed in methanol followed by addition of fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) as described (23). Cells were observed and photographed at a primary magnification of 400 X.

Determination of EGCg. EGCg was determined with the hot water extracts using the standardized chromatographic procedure described by Katiyar et al. (24). Authentic EGCg (Sigma) was used as the standard.

Materials. All chemicals were from Sigma (St. Louis, MO) unless otherwise specified. EGF was from mouse, culture grade, from Upstate Biotechnology Inc. (Lake Placid, NY). Tea infusions were prepared by sequential steeping of ca. 2-g bags of tea in 10 ml of water for 10 min each. At the end of the infusion, bags were pressed to remove liquid.

RESULTS

Epigallocatechin gallate (EGCg) was without effect on the NADH oxidase activity of plasma membrane vesicles (Fig. 1) or NADH oxidase solubilized and partially purified from the cell surface (Fig. 2) of human mammary epithelia (MCF-10A). However, with plasma membranes from human mammary adenocarcinoma (BT-20) or HeLa (human cervical carcinoma) cells, NADH oxidase activities were inhibited by 30 to 40% with an ED_{50} of about 1 nM (Fig. 1). BT-20 and HeLa cells contain a drug-responsive component of NADH oxidase activity inhibited by capsaicin or the antitumor sulfonylurea as well as NADH oxidase activities resistant to inhibition. The responses to EGCg were comparable to those for capsaicin and the sulfonylurea. Similar results were obtained with 4T1 mouse mammary cells.

With plasma membrane vesicles from the BT-20 mammary adenocarcinoma cell line, the NADH oxidase specific activity was approximately 1.5 that of the MCF-10A cell line (Fig. 1A). Upon addition of EGCg, the specific activity of the MCF-10A cells was unchanged, whereas, that of the BT-20 was reduced to approximately the same level as that of the MCF-10A cells (Fig. 1A). Also inhibited by EGCg in a similar fashion was the NADH oxidase activity from plasma membranes of HeLa cells (Fig. 1B) and 4T1 mouse mammary cells (not shown). Thus, in the plasma membrane vesicles from the BT-20, 4T1 and HeLa cells, there were both EGCg-resistant and EGCg-susceptible components whereas in the plasma membrane vesicles from the MCF 10A cells only an EGCg-resistant activity was observed (Fig. 1A).

Results similar to those observed with isolated plasma membrane vesicles were obtained as well with solubilized NADH oxidase preparations of NADH oxidase released from cultured cells by low pH treatment (Fig. 2). With BT-20 (Fig. 2A) and HeLa (Fig. 2B) preparations, activity was strongly inhibited by EGCg with an EC_{50} of between 1 and 10 nM. The released and solubilized NADH oxidase for the MCF-10A cells was much less affected by the EGCg (Fig. 2A). As with isolated plasma membrane vesicles, the specific activity of the released NADH oxidase preparations from BT-20 cells was greater (approximately twice) than that of the released preparations from MCF-10A cells. Following treatment with EGCg, the specific activity of the preparations from BT-20 cells was reduced to a level comparable to the specific activity of the preparations from MCF-10A cells. Thus, the EGCg appears to inhibit specifically the drug-responsive NADH oxidase component of the tumorigenically transformed cell lines but not that of the constitutive NADH oxidase activity of the MCF-10A mammary epithelial line.

EGCg also inhibited the growth of the BT-20 and 4T1 mammary adenocarcinomas and HeLa cells in culture (Fig. 1C, D). While not as striking as for the inhibition of NADH oxidase, EGCg did preferentially restrict the growth of the HeLa, BT-20and 4T1 cells compared to MCF-10A (Fig. 1C, D). Growth of the MCF-10A mammary epithelial cells was unaffected by EGCg except at very high doses of 100 μM (Fig. 1C), whereas that of the tumorigenically transformed BT-20, 4T1 and HeLa cells was 50% inhibited at about 5 μM (Fig. 1C, D).

Despite early growth inhibition of MCF-10A cells by EGCg, the cells quickly recovered and eventually grew normally (Fig. 3). This is in contrast to HeLa, 4T1 and BT-20 cells where the cells did not recover and died (Fig. 3).

Measurements of the diameters of treated HeLa and BT-20 cells taken directly from printed micrographs revealed that, on average, the cells treated with 5 to 50 μM EGCg exhibited volumes ≈ 50% those of untreated cells. At 1μM EGCg, there was no response of any of the cell lines at 72 h despite the fact that this EGCg concentration inhibited the tNOX activity of isolated plasma membranes. The possibility was considered that the combination of a reversible inhibition and rapid metabolism of EGCg might result in an overall lack of growth inhibition at 1 μM EGCg after 3 d. To test this possibility, cells were treated with 1 μM EGCg twice daily for 96 h after which time the cells were photographed, measured and counted. Cell diameters were reduced on average by about 25% and cell volume by 50% by the twice daily 1 μM EGCg dosage. Cell number also was reduced by about 25% with both HeLa and BT-20 cells by the 1 μM EGCg provided twice daily whereas with the non-cancer MCF-10A cells, growth rate and cell diameters were unaffected or slightly increased. When the cells treated with 10 or 50 μM EGCg were stained to reveal DAPI fluorescence, a very large percentage of the treated cells showed nuclear DNA with the condensed and fragmented appearance characteristic of apoptotic cells (Fig. 4).

Since EGCg is considered as one of the major compounds contributing to the cancer preventative actions attributed to green tea (11-16), green tea infusions were examined as well for their ability to inhibit the NADH oxidase. Both the solubilized and partially purified NADH oxidase released from cells by low pH treatment (Fig. 5) and the NADH oxidase of sera pooled from cancer patients (Table 1) were inhibited by green tea infusions. Infusions of green tea were approximately ten times more effective than those of black tea and correlated approximately with the content of EGCg with an EC_{50} of 2 μM EGCg equivalent to 1 μg/ml.

DISCUSSION

Our laboratory has identified an NADH oxidase activity of the plasma membrane. In rat liver (1-3), keratinocytes (6), and plant stems (25), the activity was stimulated by hormones and growth factors. With rat hepatoma plasma membranes, the activity was constitutively activated and no longer hormone responsive (4, 5). The NADH oxidase activity of rat liver plasma membranes required quinones for activity (8). Stimulation or inhibition of the activity has correlated closely with inhibition or stimulation of growth (26, 27).

Subsequent studies showed the NADH oxidase of plasma membranes of tumorigenically transformed cell lines to exhibit an activity responsive to a small number of antitumor or putative antitumor substances including the antitumor sulfonylureas (10) and the vanilloid quinone site inhibitor capsaicin (9). Plasma membranes from cell lines not tumorigenically transformed did not respond to these drugs nor was the growth of cell lines not tumorigenically transformed affected by these drugs.

In the present report, we demonstrate that the NADH oxidase activity of human mammary adenocarcinoma cells and HeLa cells (human cervical carcinoma derivation) were inhibited by epigallocatechin gallate, a catechin present in high concentrations in green tea and thought to be one of the principal compounds responsible for the anticancer activities attributed to green tea (12). In contrast to the NADH oxidase of the plasma membranes of the carcinoma cell lines, the NADH oxidase of plasma membranes from mammary epithelial cells was not inhibited.

Animal *in vivo* studies (11-14, 16) and human epidemiological observations (11, 13, 15) have indicated anticancer effects for tea. In general, these effects have been attributed to epigallocatechin gallate (EGCg) a major tea catechin (12, 24). Other antioxidant catechins and polyphenols present in tea have less marked anticancer properties. The activity is related in some measure to regulation of cell cycle progression and induction of p^{53}-dependent apoptosis (28).

Not only did EGCg inhibit the NADH oxidase of plasma membrane vesicles from cancer cells and not that of normal cells, the substance exerted a parallel response on growth. Growth of HeLa cells was almost completely inhibited by EGCg whereas growth of CHO cells and mammary epithelial cells was much less affected by EGCg. With treated HeLa cells, nuclei exhibited patterns of fluorescence characteristic of apoptosis (22). Thus, the cyanide-resistant NADH oxidase of the plasma membrane appears to represent an enzymatic activity whose inhibition by EGCg correlates with an inhibition of growth and subsequent apoptosis in susceptible cancer cell lines.

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Table 1. Inhibition of tNOX activity by tea infusions and by epigallocatechin gallate (EGCg), the major tea polyphenol (catechin) of green tea, for sera pooled from patients with cancer. The EGCg content was determined as described (22). Results were repeated 3 to 5 times with different sources and preparations of both black and green tea and with consistent findings.

Source	EC_{50}	EGCg μg/ml
Black tea	1:10 to 1:100	1
Green tea	1:1000	1
Epigallocatechin gallate (EGCg)	2 μM	1

LIST OF FIGURES

Cells were grown on coverslips in the absence (upper panel) or presence (lower 2 panels) of 10 or 50 μM EGCg and fixed. Nuclear DNA was stained with DAPI and analyzed with a fluorescence microscope.

Fig. 5. Inhibition of partially purified tNOX from HeLa cells by green tea infusions. The EC_{50} for inhibition of the enzymatic activity was at a tea dilution of about 1:1000. The preparations contained an activity resistant to inhibition as well so that the inhibition by the tea infusions was not complete and further inhibition by green tea was not observed above a dilution of about 1:10. Results are averages of duplicate determinations in each of three separate experiments (n = 6) ± standard deviations among experiments (n = 3).

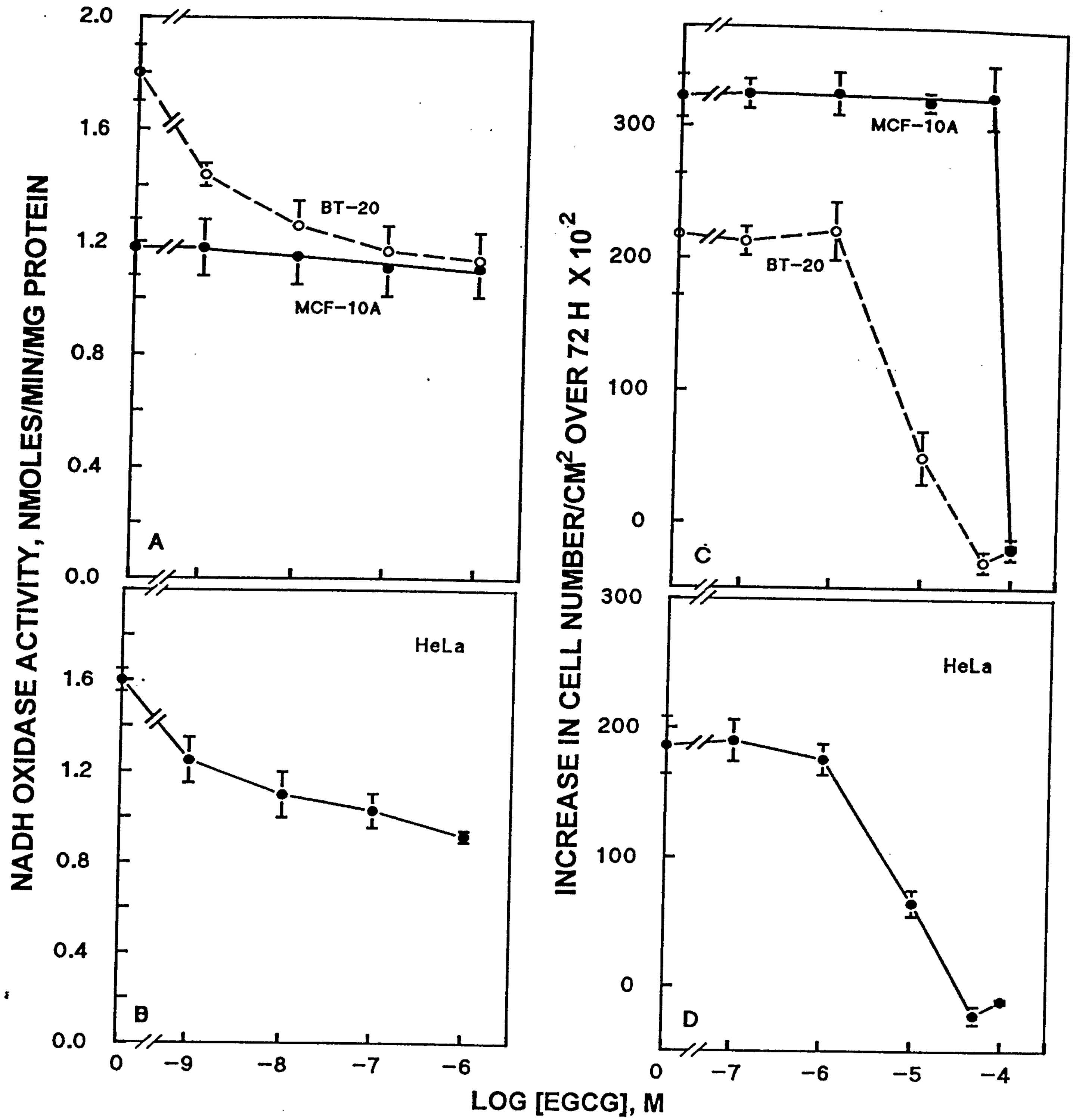

NADH OXIDASE ACTIVITY, NMOLES/MIN/MG PROTEIN
2.0
1.6
1.2
0.8
0.4
0.0
BT-20
MCF-10A
A
HeLa
B
0
-9
-8
-7
-6
INCREASE IN CELL NUMBER/CM^2 OVER 72 H X 10^2
300
200
100
0
MCF-10A
BT-20
C
HeLa
D
-7
-6
-5
-4
LOG [EGCG], M

Fig. 1

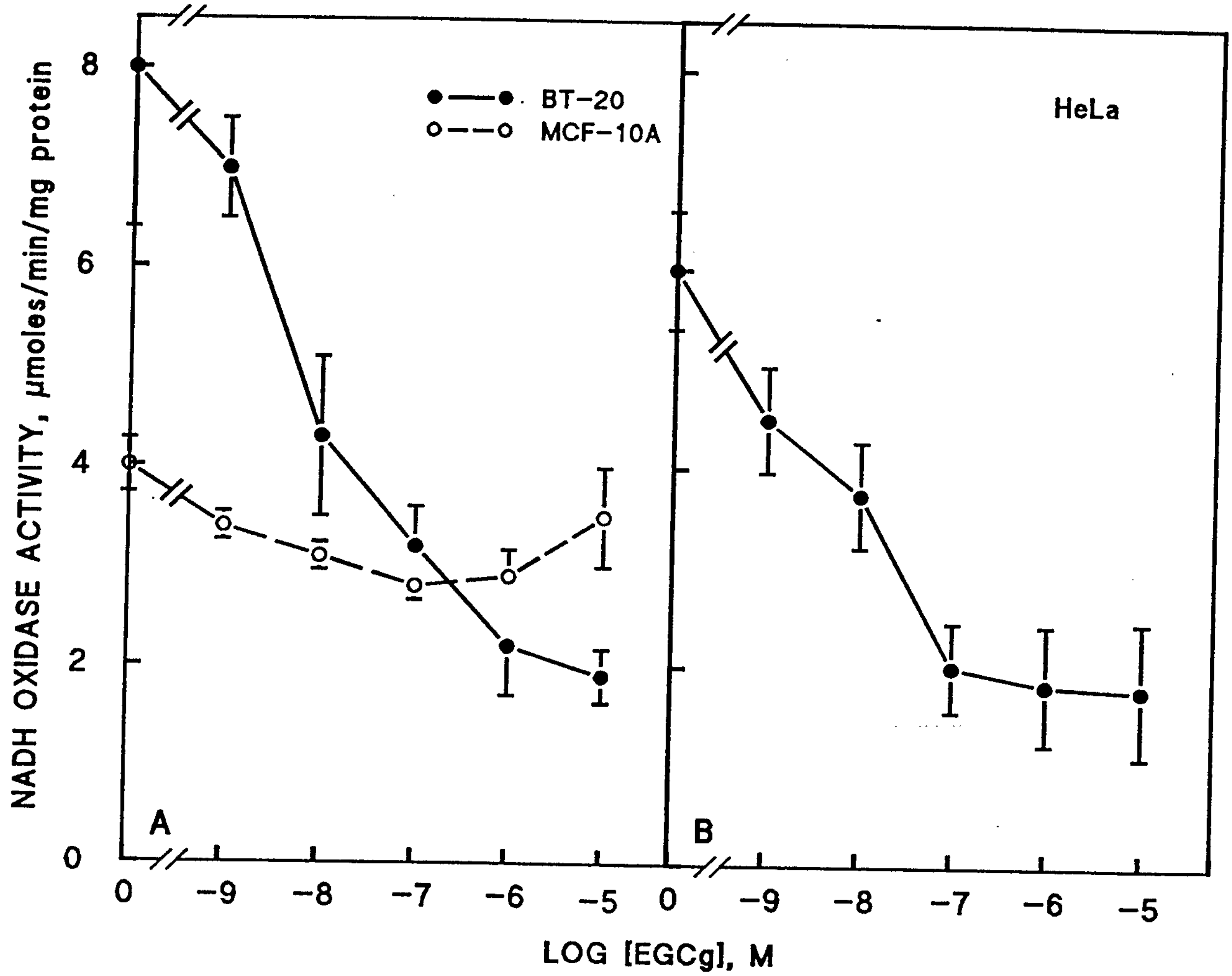

NADH OXIDASE ACTIVITY, µmoles/min/mg protein
8
6
4
2
0
BT-20
MCF-10A
HeLa
A
B
0
-9
-8
-7
-6
-5
0
-9
-8
-7
-6
-5
LOG [EGCg], M

Fig. 2

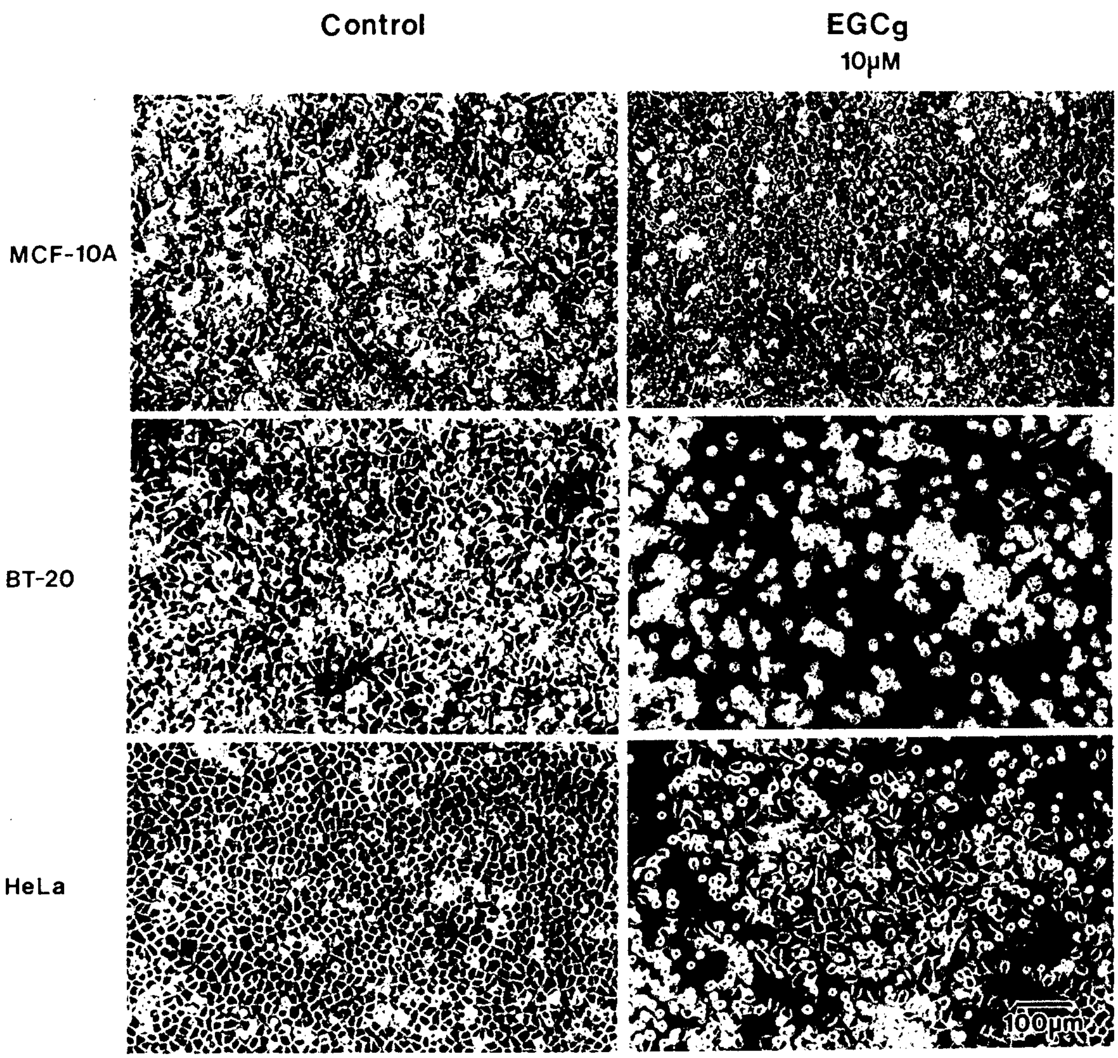


Fig. 3

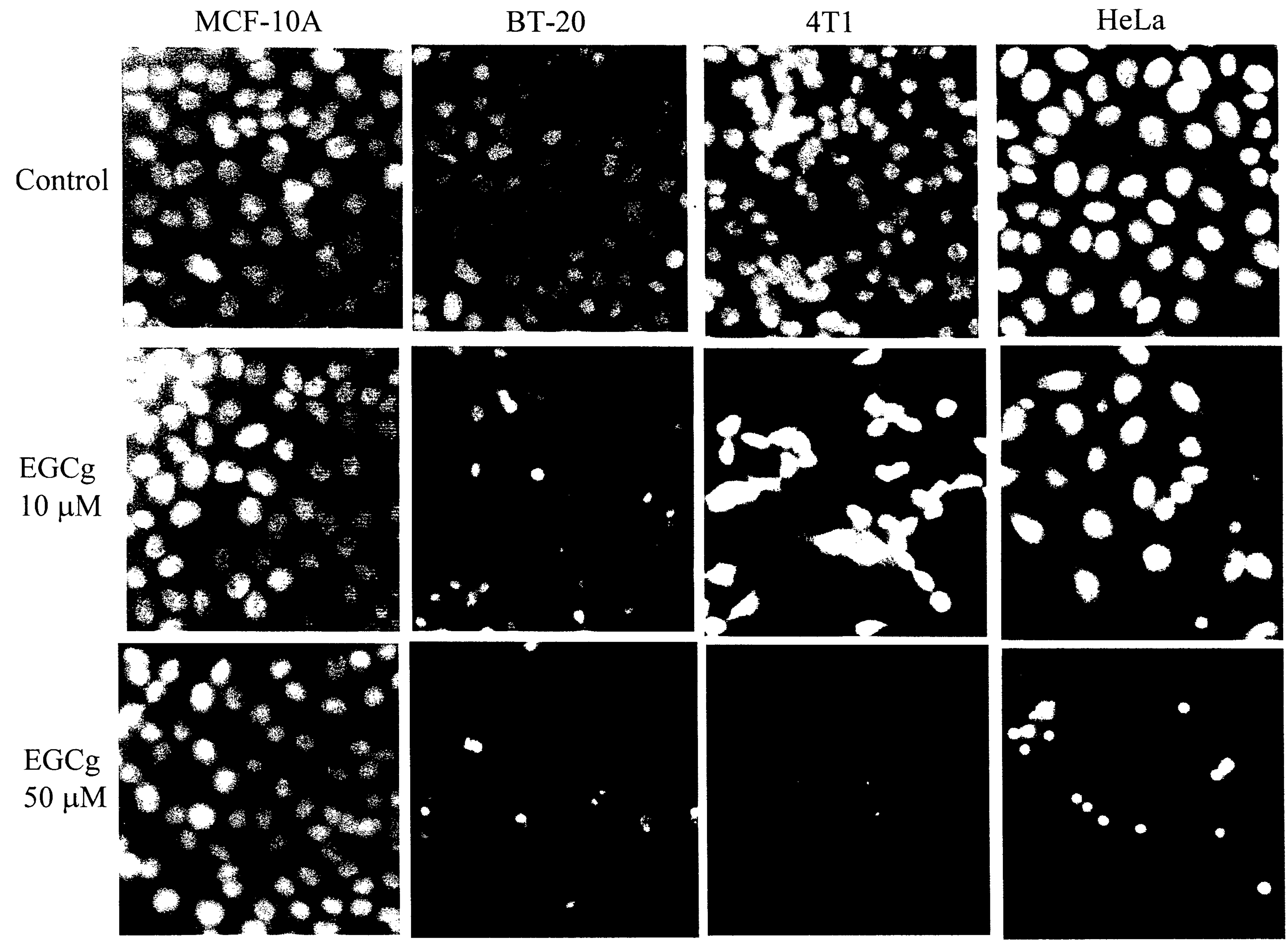
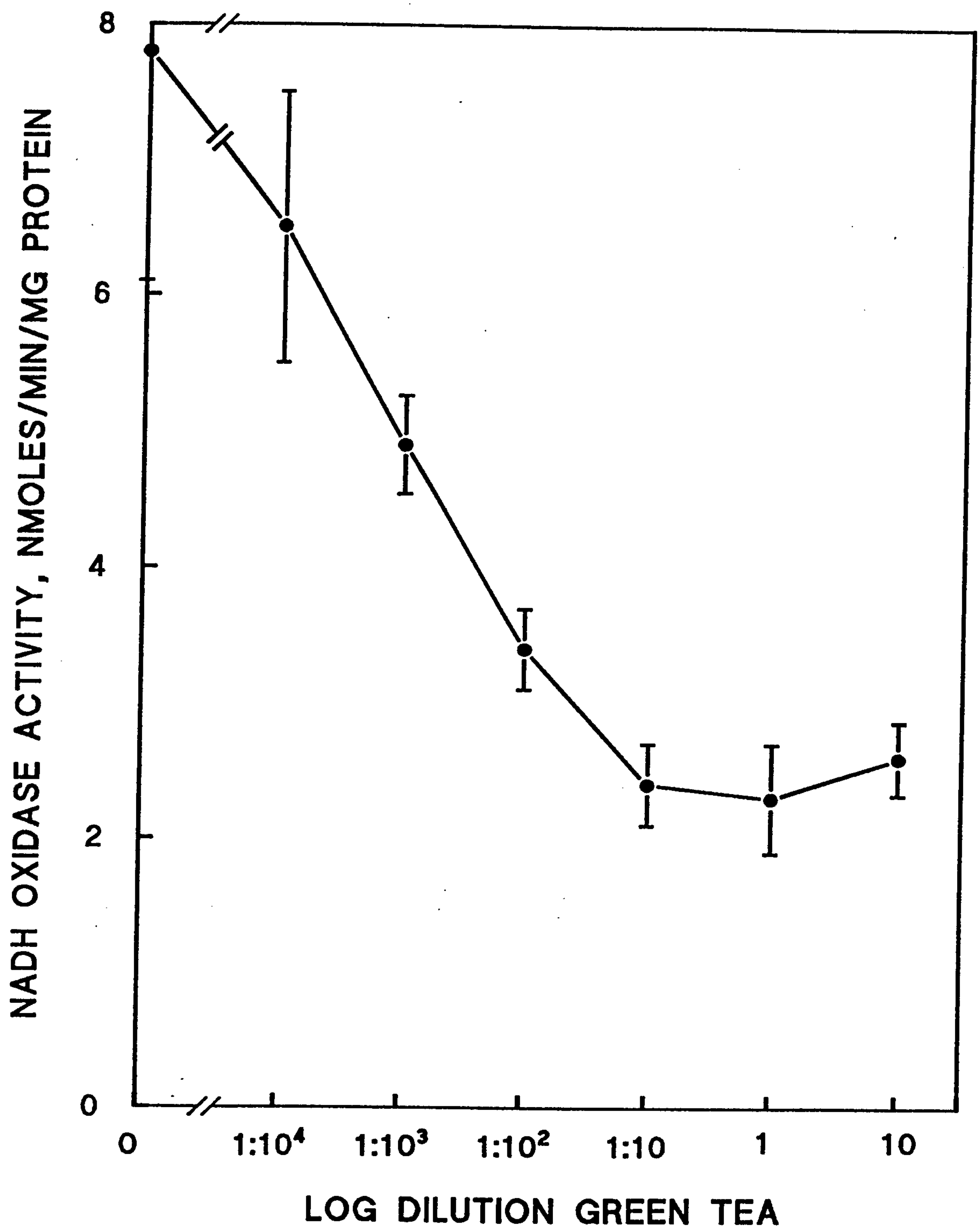


Fig. 4



NADH OXIDASE ACTIVITY, NMOLES/MIN/MG PROTEIN
8
6
4
2
0
0
1:10⁴
1:10³
1:10²
1:10
1
10
LOG DILUTION GREEN TEA

Fig. 5

ACTIVITY OF THE ANTICANCER QUASSINOID, GLAUCARUBOLONE, ENHANCED BY TEA CATECHINS

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Summary

The anticancer quassinoid, glaucarubolone, inhibits both growth and a plasma membrane NADH oxidase associated with the surface of cancer cells. The inhibition is enhanced by tea catechins with a crude mixture being more effective than the principal anticancer catechin, epigallocatechin gallate (EGCg). A 3-way mixture of epicatechin (EC) EGCg and glaucarubolone was more effective in inhibiting the cancer-associated NADH oxidase and growth of both HeLa and 4T1 mouse metastatic breast cancer cells than were individual components of the mixtures or individual components added two at a time. Neither EGCg nor EC affects the growth of nontransformed cell lines in culture (human mammary epithelia MCF-10A). The results offer an opportunity to utilize relatively safe and non-toxic tea catechins to enhance the response of tumor cells to an anticancer drug targeted to the plasma membrane NADH oxidase of the cell surface.

Key Words: quassinoids, antitumor agents, growth inhibition, glaucarubolone, NADH oxidase, plasma membranes, HeLa cells, tea catechins, epigallocatechin gallate, mouse mammary cancer 4T1 cells.

A drug-responsive NADH oxidase has been described from our laboratory (1). The activity is inhibited by several known or potential antitumor agents including the antitumor diarylsulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) (2), the vanilloid capsaicin (3), adriamycin (4), the antitumor acetogenin bullatacin (5), the anticancer quassinoid, glaucarubolone (6) as well as impermeant conjugates of LY181984 (7), glaucarubolone (6), the vanilloids (8) and of adriamycin (4). The drug responsive NADH oxidase is not observed with plasma membrane vesicles of non-transformed cells (1, 3).

There has been several reports of the use of tea catechins to enhance the activity of doxorubicin (adriamycin) (9, 10) and other known antitumor agents (11). Epigallocatechin gallate (EGCg) is also a very effective inhibitor of the cancer-associated plasma membrane NADH oxidase and is virtually without effect on either growth or the plasma membrane-associated NADH oxidase of normal cells. When combined with glaucarubolone, they exert an enhancing effect of several log orders greater than that achieved by either drug alone.

In this report, we demonstrate that the NADH oxidase of the plasma membrane may also be a target for the antitumor action of the principal tea catechin, epigallocatechin gallate (EGCg) (12). A potentiating action of EGCg plus epicatechin (EC) with glaucarubolone both on the inhibition of the plasma membrane NADH oxidase and of cancer cell growth similar to that observed with adriamycin (9, 10) is demonstrated.

Materials and Methods

Growth of cells. HeLa (ATCC CCL2) cells were grown in 150 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37°C with 10% bovine calf serum (heat-inactivated), plus 50 mg/l gentamicin sulfate (Sigma). Cells were trypsinized with Sigma IX trypsin for 1 to 2 min and harvested by scraping and taken up in 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4, to a final cell concentration of 0.1 g wet weight (gww) per ml.

A mouse mammary tumor subpopulation line 4T1 arising from a BALB/cf C3H mouse (12, 13) was grown in DME-10, Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% newborn calf serum, 1 mM mixed non-essential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Purification of plasma membranes from cultured cells. Cultured cells were collected by centrifugation for 6 to 15 min at 1,000 to 3,000 rpm. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron Homogenizer for 30 to 40 sec at 10,500 rpm using a PT-PA 3012/23 or ST-probe in 7 to 8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 175 g to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 g□min (e.g., 1 h at 23,500 g) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ration of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis. The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w/ Polyethylene Glycol 3350 (Fisher), and 5 mM potassium phosphate, pH 7.2 (14). The homogenate (1 g) was added to the two-phase system and the weight of the system was brought to 8 g with distilled water. The tubes were inverted vigorously for 40 times in the cold (4°C). The phases were separated by centrifugation at 750 rpm (150 x g) in a Sorvall HB 4 rotor for 5 min. The upper phases were withdrawn carefully with a Pasteur pipette, divided in half and transferred into 40 ml plastic centrifuge tubes. The tube contents were diluted with cold 1 mM $NaHCO_3$ and collected by centrifugation at 10,000 x g in a HB rotor for 30 min. Plasma membrane pellets were resuspended in 50 mM Tris-Mes buffer (pH 7.2) and stored at -70°C. Proteins were determined using the bicinchoninic acid (BCA) assay (14) with bovine serum albumin as standard. The upper phase, enriched in plasma membranes, was diluted

5-fold with 1 mM sodium bicarbonate and the membranes are collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Spectrophotometric assay of NADH oxidase. NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN, and 150 μM NADH at 37°C. Activity was measured using a Hitachi U3210 spectrophotometer with stirring and continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity.

Glaucarubolone and glaucarubolone-aminopolyethyloneglycol conjugate. Glaucarubolone (I, Fig. 1) was isolated from root bark of *Castela polyandra* as described (17). For stereospecific conjugation via the $C_{(15)}$ hydroxyl group, we prepared II (Fig. 1) using a procedure that differentiated the $C_{(1)}$, $C_{(12)}$ and $C_{(15)}$ hydroxyl groups in glaucarubolone. Exposure of glaucarubolone to TMSOTf in pyridine containing triethylamine (0°C, RT, 1 h) gave rise (75% yield) to tris-trimethylsilylated material III (Fig. 1) in which the $C_{(12)}$ hydroxyl was exposed. Exposure of III to tetrabutylammonium fluoride in THF cleaved exclusively in excellent yield the $C_{(15)}$ OTMS ether. Treatment of IV (Fig. 1) with succinic anhydride in methylene chloride containing trimethylamine and 4-

dimethylaminopyridine gave rise (80%) after treatment with HF/CH_3CN to II (Fig. 1) which was then conjugated with amino polyethyleneglycol. Note that the $C_{(12)}$ hydroxyl was sufficiently hindered to allow stereospecific acylation of the $C_{(15)}$ hydroxyl giving rise exclusively to II (Fig. 1). The derivatized glaucarubolone (II, Fig. 1) was coupled to amino polyethyleneglycol (Ave MW 5,000) in the presence of 10 mM of the coupling reagent dicyclohexylcarbodiimide (DCC) (Sigma).

Results

The anticancer quassinoid glaucarubolone in the presence or absence of 100 μM epigallocatechin gallate (EGCg) inhibited the oxidation of NADH by vesicles of plasma membrane isolated from HeLa cells (Fig. 1). However, an equivalent amount of EGCg supplied as a crude decaffeinated tea extract (Tegreen) was considerably more effective in enhancing the inhibition of NADH oxidation by glaucarubolone (Fig. 1). Similar results were obtained with the polyethyleneglycol (PFG) conjugate of glaucarubolone (Fig. 2). The conjugate was more effective than the free glaucarubolone but equivalent amounts of EGCg supplied as Tegreen was more effective than EGCg alone in potentiating the response to glaucarubolone.

To determine if an interaction among one or more tea catechins could account for the enhanced response of the HeLa cell plasma membrane NADH oxidase to glaucarubolone when treated in the presence of Tegreen compared to EGCg, mixtures of EGCg with EC were tested first with a NOX preparation solubilized from HeLa cells (Fig. 3) and subsequently with plasma membrane (Fig. 4).

With the solubilized NOX protein, maximum inhibition was achieved by a mixture of 1 nM EGCg plus 50 μM EC. Neither EC alone (including 100 μM) nor 1 nM EGCg were effective in inhibiting the activity of the solubilized plasma membrane NADH oxidase protein.

Cell killing was considerably enhanced by the combination of EGCg and EC (Fig. 4). In the absence of EC, 50% growth arrest by EGCg was observed at 10 μM. However, in the presence of 100 μM EC, the concentration of EGCg for 50% growth arrest was lowered to 0.1 μM and in one experiment, the cells were totally killed by the combination of 0.1 μM EGCg plus 100 μM EC.

A similar response was seen with the NADH oxidase activity of isolated plasma membrane vesicles in the presence of glaucarubolone (Fig. 5). With 100 μM EC, 100 μM EGCg or no addition, the response to glaucarubolone was minimal. However, when 0.1 μM EGCg was combined with 100 μM EC, a substantial dose response to glaucarubolone was observed.

Mouse 4T1 mammary carcinoma cells are particularly refractory to drug-induced growth inhibition and cell killing. They are largely unaffected by glaucarubolone

(Table 1). However in the presence of the combination of 0.1 μM EGCg and 100 μM EC, the cells were completely killed at 1 μM glaucarubolone (Table 1). This remarkable drug response was reflected in the inhibition of the oxidation of NADH by the intact 4T1 cells (Table 2) (Fig. 6). The activity was completely inhibited back to basal levels by 1 μM glaucarubolone in the presence of 0.1 μM EGCg plus 100 μM EC. The EC_{50} of glaucarubolone for inhibition of the drug-responsive component of the plasma membrane NADH oxidase was 2 nM whereas with glaucarubolone alone or in the presence of 100 μM EC alone 0.1 μM EGCg alone or 100 μM EC + 0.1 μM EGCg, the EC_{50} of glaucarubolone for inhibition was between 20 and 50 nM (Table 2).

Discussion

Previous work has suggested that tea catechins may enhance the action of antitumor drugs including adriamycin. The basis for this enhancement has not been investigated.

Our work previously has identified a growth-related protein of the cell surface, a hydroquinone (NADH) oxidase (18) with protein disulfide thiol interchange activity designated as the NOX protein.

With cancer cells and tissues, the plasma membrane-associated oxidation of NADH was responsive to a small group of drugs with anticancer or potential anticancer activity whose modes of action were either unknown or suspected to involve a cell surface site. Included were an impermeant drug conjugate of adriamycin (4), an antitumor sulfonylurea (2, 19) and an impermanent sulfonylurea conjugate inhibitory to growth of HeLa cells (7), the antitumor acetogenin bullatacin (5), the vanilloid capsaicin (3, 8), glaucarubolone, a quassinoid with anticancer activity (6) and an impermeant conjugate of glaucarubolone with polyethylene glycol (6).

Quassinoids derive their name from the parent compound quassin first identified as a bitter principle from plants of the family Simaroubaceae (20, 21). Glaucarubolone was selected for initial testing due to the presence of a free $C_{(15)}$ hydroxyl to allow conjugation to form an impermeant derivative to test the concept of a cell surface site of action.

Glaucarubolone has been shown previously to have therapeutic activity in vivo against C-38, a transplantable murine colon carcinoma with a % T/C of 14 and a MTTD of 151 mg/kg body weight (22). Glaucarubolone also was active in vitro on L1210 lymphocyte leukemia and human CX-1 colon carcinoma human cervical carcinoma (HeLa) and Kaposi's sarcoma cells (6).

A cell surface site for the drug-responsive NADH oxidase has been established for the antitumor sulfonylureas (7, 23). Similar indications were provided here for glaucarubolone (6). By conjugating glaucarubolone to amino PEG, activity both in inhibiting the plasma membrane NADH oxidase and in inhibiting the growth of HeLa cells were retained. Within the same experiment, the conjugate on a sulfonylurea basis was approximately equivalent to free glaucarubolone in inhibiting the NADH oxidase and superior to free glaucarubolone in inhibiting the growth of HeLa cells. The plasma membrane vesicles used in these experiments were sealed and right side-out (23). Therefore, for the glaucarubolone-amino PEG conjugate to have inhibited the drug-responsive NADH oxidase activity with the same efficiency as free glaucarubolone, a drug-responsive NADH site at or near the cell surface was implied.

As the NADH site inhibited by glaucarubolone is located on the external surface of the cell, NADH is unlikely to be a natural substrate. The natural electron donor for the activity of HeLa cells has been shown to be hydroquinone of the plasma membrane (18). Hydroquinone oxidation shows a similar response to the antitumor sulfonylureas and capsaicin as does the oxidation of NADH (18). Yet, NADH oxidation, as a convenient measure of the cancer-specific, drug responsive activity of the plasma membrane of transformed cells and tissues.

With the antitumor sulfonylurea LY181984 and with capsaicin, the activity both in terms of inhibition of HeLa cell growth and in inhibition of the plasma membrane NADH oxidase was very much dependent upon redox state (24, 25). For the antitumor sulfonylureas, activity was enhanced under reducing conditions (24) whereas, for capsaicin, oxidizing conditions were required for maximum activity (25). With glaucarubolone, the inhibition was favored under oxidizing conditions especially for the conjugate where the EC_{50} for activity was lowered substantially in the presence of 100 μM oxidized glutathione compared to reduced glutathione (6). The enhanced activity in the presence of oxidized glutathione also was seen as an enhanced inhibition of HeLa cell growth especially by the glaucarubolone conjugate under the oxidizing conditions afforded by GSSG. With capsaicin, the inhibition of growth of human and mouse melanoma cells in vitro and of mouse melanoma in vivo also was dependent upon oxidizing conditions (25). These findings suggest the possibility of therapeutic benefit both from immobilization of glaucarubolone to restrict its action to cell surface sites and from modification of the redox environment by means of co-administration to the tumor site of glaucarubolone or glaucarubolone-conjugates and mild oxidizing agents such as oxidized glutathione or *tert*-butylhydroperoxide (25). However, it is unlikely that the response to tea catechins is via this mechanism.

In this study, the response of the plasma membrane NADH oxidase of plasma membrane vesicles was quite variable even under conditions where the redox environment was fully oxidized with hydrogen peroxide. The EC_{50} was observed to vary by several orders of magnitude.

The explanation for this unusual dose-response and the unusual dose-response with the conjugate between 1 nM and 1 μM under oxidizing conditions (25) may derive from the phenomena of multiple activity forms of the NADH oxidase associated with HeLa cell plasma membranes (1). Some are drug responsive and constitutively activated in cancer whereas others are less drug unresponsive and present in both malignantly transformed and non-transformed cells and tissues (3, 19). It may be that different preparations contain different proportions of the cancer-specific, drug-responsive activity form. The molecular basis for the differential drug response in response to redox environment is unknown but appears to be related to the facility with which the target NADH oxidase is able to bind the drug. One possibility under investigation is that the tea catechins alter the ability of the NOX protein to bind glaucarubolone and other anticancer drugs targeted to the NADH oxidase. This is supported by the observations with the mouse metastatic breast cancer cell line, 4T1. Growth and NADH oxidase activity of this cell line is normally refractory to inhibition by both glaucarubolone and by EGCg. However, the combination of the three agents, glaucarubolone, EGCg and EC, resulted in substantial cell killing at submicromolar concentrations of both glaucarubolone and EGCg.

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Table 1. Killing of 4T1 metastatic mouse mammary cancer cells.

Addition	Increase in cell number cm^{-2} over 72 h • 10^2
None	550
Glaucarubolone 1 μM	450
EGCg 0.1μM	520
EC 100 μM	560
Glaucarubolone 1 μM + EGCg 0.1 μM	405
Glaucarubolone 1 μM + EGCg 0.1 μM + EC 100 μM	-40*

*100% Dead

Table 2. EC_{50} of glaucarubolone inhibition of the cell surface NADH oxidase of 4T1 mouse mammary carcinoma cells in the presence of 100 μM epicatechin (EC) or 0.1 μM epigallocatechin gallate (EGCg) alone or in combination. NADH oxidation was determined for 10^6 cells with 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M glaucarubolone. The drug responsive NOX activity was maximally inhibited at 10^{-5} M glaucarubolone. Results are means from 3 experiments.

Addition	EC_{50}
None	2×10^{-8} M
100 μM EC	1×10^{-8} M
0.1 μM EGCg	2×10^{-7} M
0.1 μM EGCg + 10 μM EC	8×10^{-9} M
0.1 μM EGCg + 100 μM EC	2×10^{-9} M

List of Figures

Fig. 1. Dose response of the NADH oxidase activities of plasma membrane vesicles from HeLa cells to glaucarubolone in the presence of the principal tea catechin epigallocatechin gallate (EGCg) and a crude decaffeinated mixture of tea catechins obtained commercially (Tegreen). Values are based on duplicate determinations averaged from each of three experiments ± standard deviations.

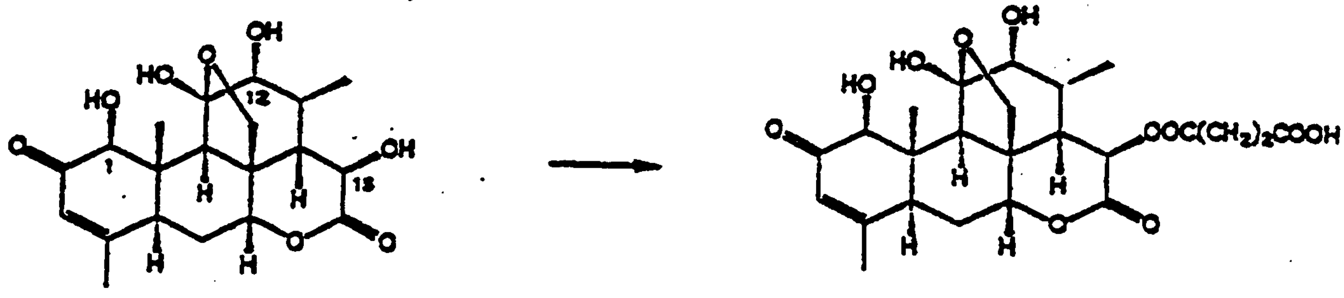
Fig. 2. As in Figure 1 except that the glaucarubolone was provided as a conjugate with polyethylene glycol (Ave. MW 5,000) (PEG). Values are based on duplicate determination averaged from each of three experiments ± standard deviations.

Fig. 3. Response of the NADH oxidase activity solubilized and partially purified as described (25) from plasma membrane vesicles of HeLa cells to 1 nM epigallocatechin gallate (EGCg) alone and in combination with epicatechin (EC) at 10, 50 and 100 μM. Values are from duplicated determinatins from each of three different experiments ± standard deviations.

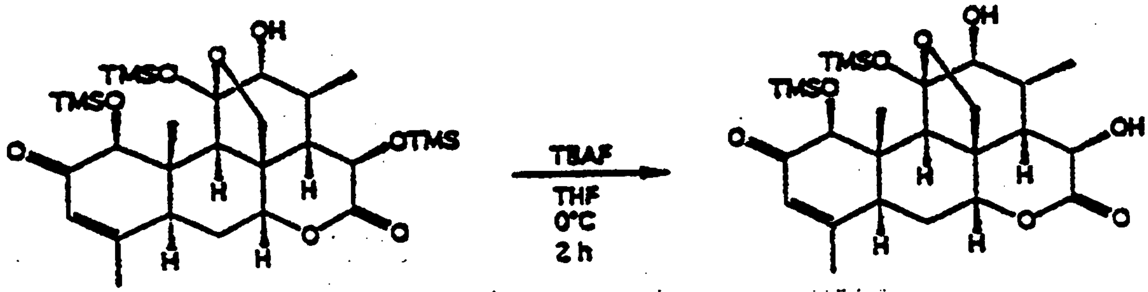
Fig. 4. Dose response of the growth of HeLa cells to epigallocatechin (EGCg) in the absence and presence of epicatechin (EC). Values are from duplicate determinations from single experiments except for 10^{-7} M EGCg which is the average of duplicate determinations from 3 experiments ± standard deviations.

Fig. 5. Response of the NADH oxidase activity of HeLa plasma membranes to varying concentrations of glaucarubolone in the absence (no addition) and presence of 100 μM epicatechin (EC) and a mixture to the two. Results are from duplicate determinations within a single experiment.

Fig. 6. Response of the NADH oxidase of 4T1 mouse mammary cells to glaucarubolone in the presence of a mixture of 0.1 μM epigallocatechin gallate (EGCg) plus 100 μM epicatechin (EC).



I II



III IV

Text Figure

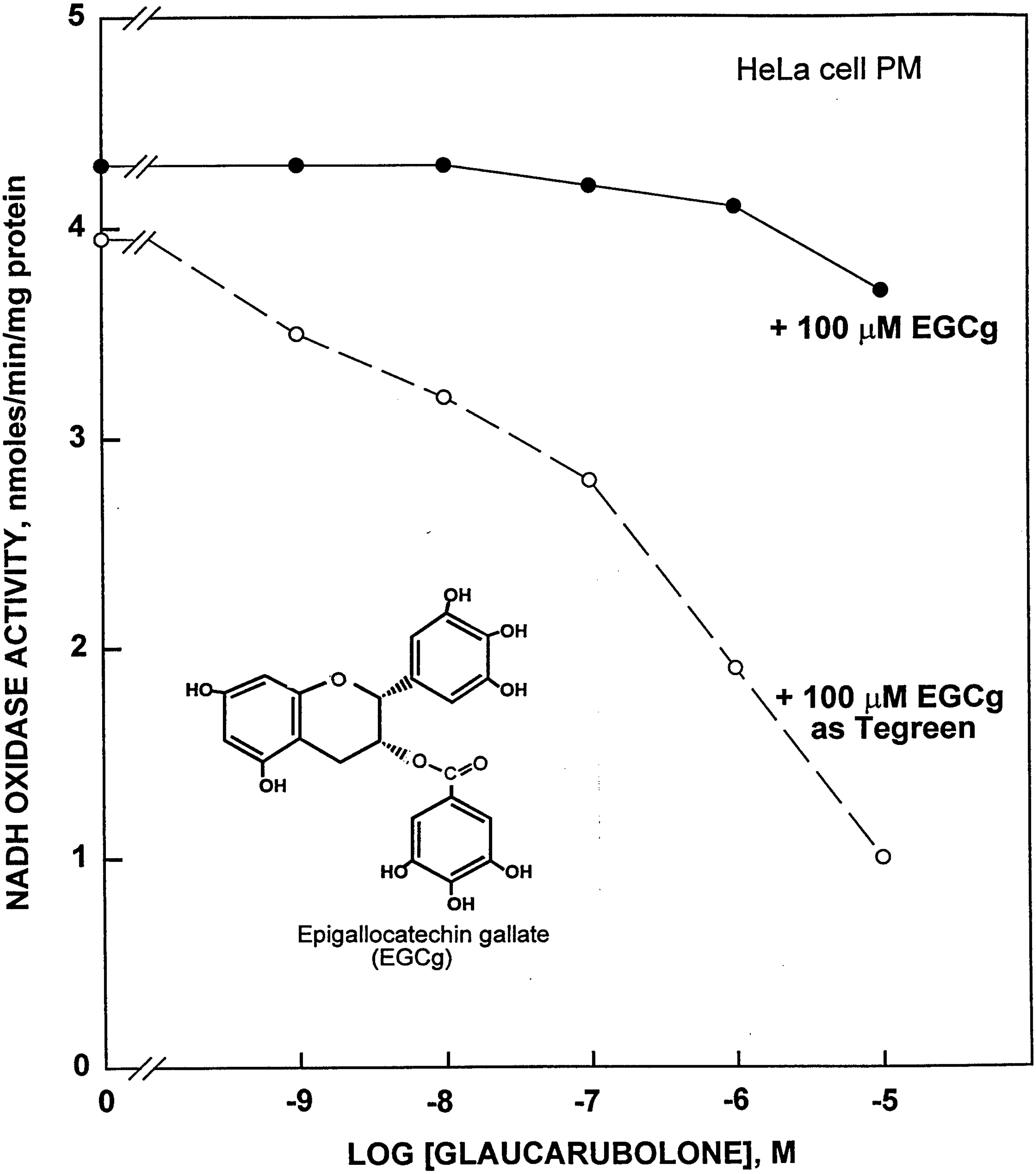

HeLa cell PM
+ 100 µM EGCg
+ 100 µM EGCg as Tegreen
Epigallocatechin gallate (EGCg)
NADH OXIDASE ACTIVITY, nmoles/min/mg protein
LOG [GLAUCARUBOLONE], M
0
1
2
3
4
5
0
-9
-8
-7
-6
-5

Fig. 1

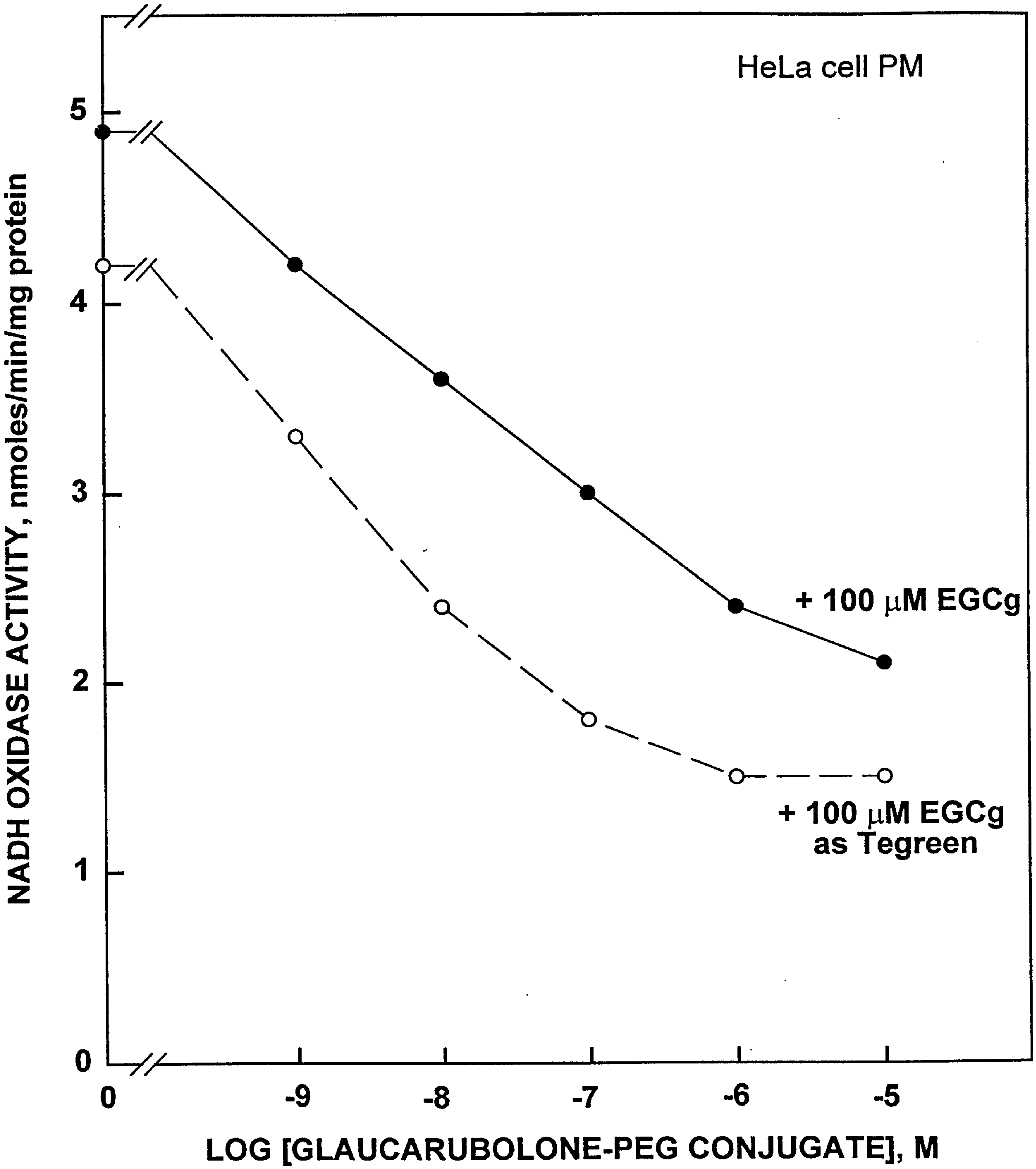

HeLa cell PM
NADH OXIDASE ACTIVITY, nmoles/min/mg protein
5
4
3
2
1
0
0
-9
-8
-7
-6
-5
LOG [GLAUCARUBOLONE-PEG CONJUGATE], M
+ 100 μM EGCg
+ 100 μM EGCg
as Tegreen

Fig. 2

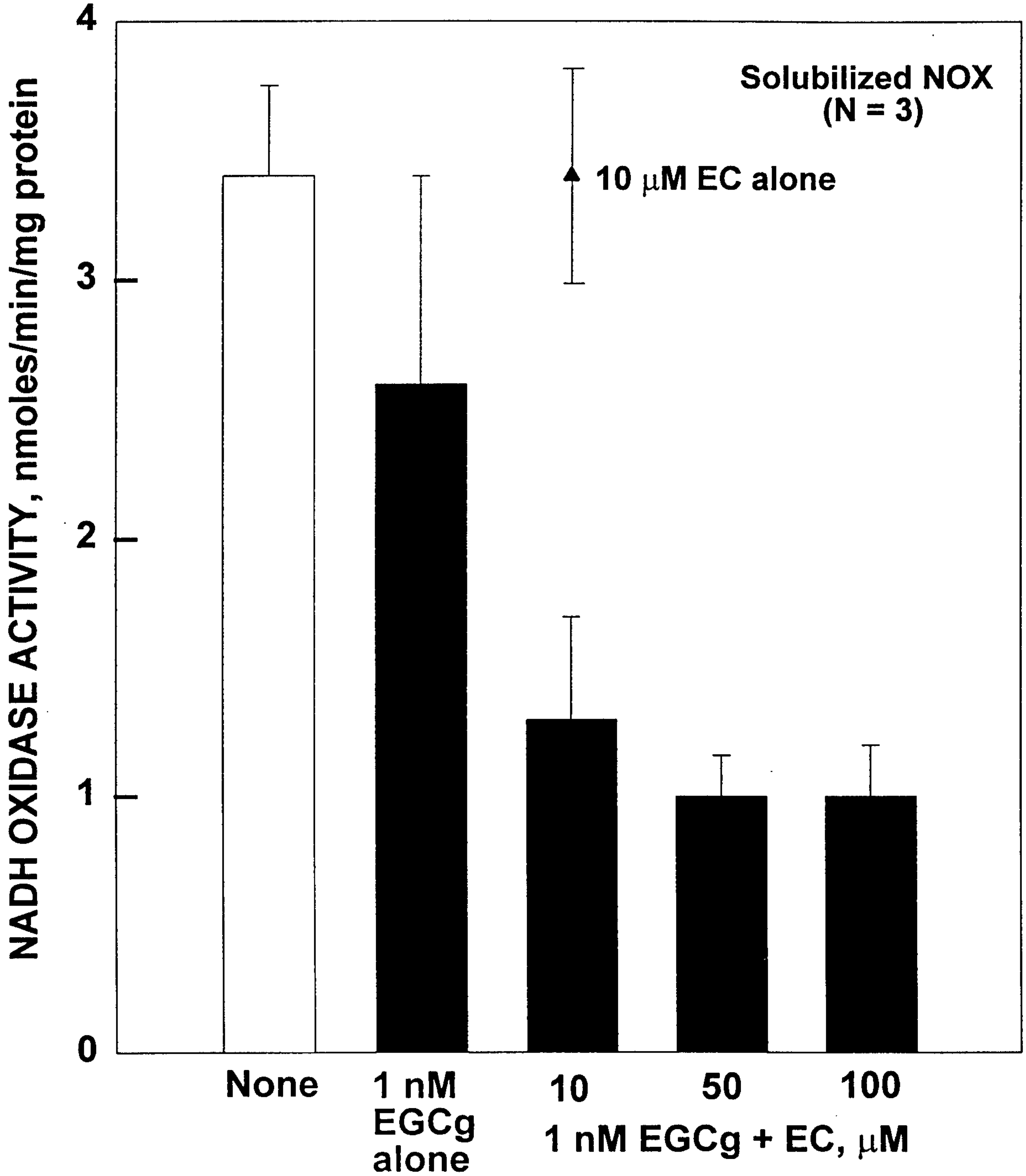

NADH OXIDASE ACTIVITY, nmoles/min/mg protein
4
3
2
1
0
Solubilized NOX
(N = 3)
10 μM EC alone
None
1 nM EGCg alone
10
50
100
1 nM EGCg + EC, μM

Fig. 3

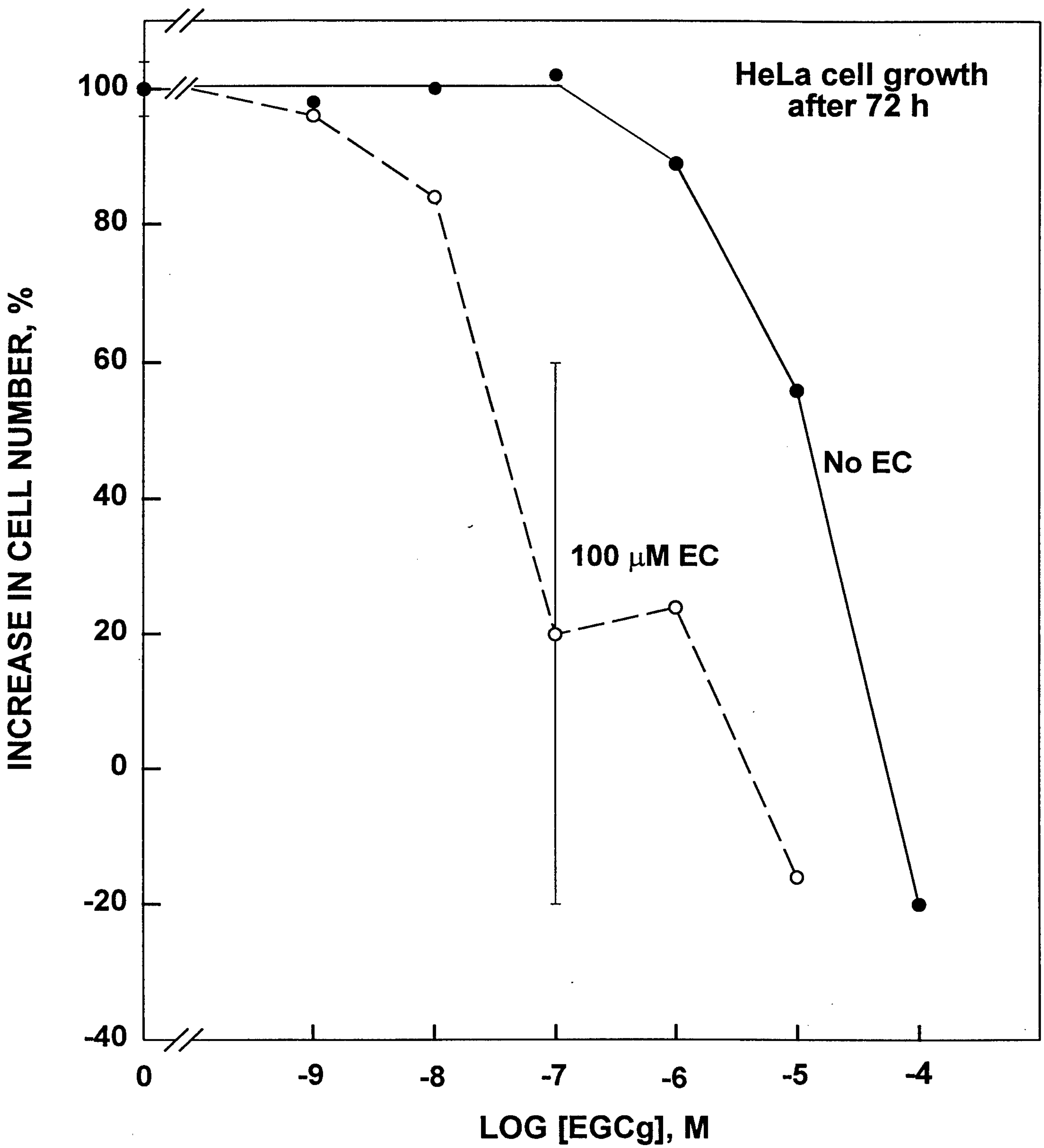

HeLa cell growth
after 72 h
INCREASE IN CELL NUMBER, %
100
80
60
40
20
0
-20
-40
0
-9
-8
-7
-6
-5
-4
LOG [EGCg], M
No EC
100 μM EC

Fig. 4

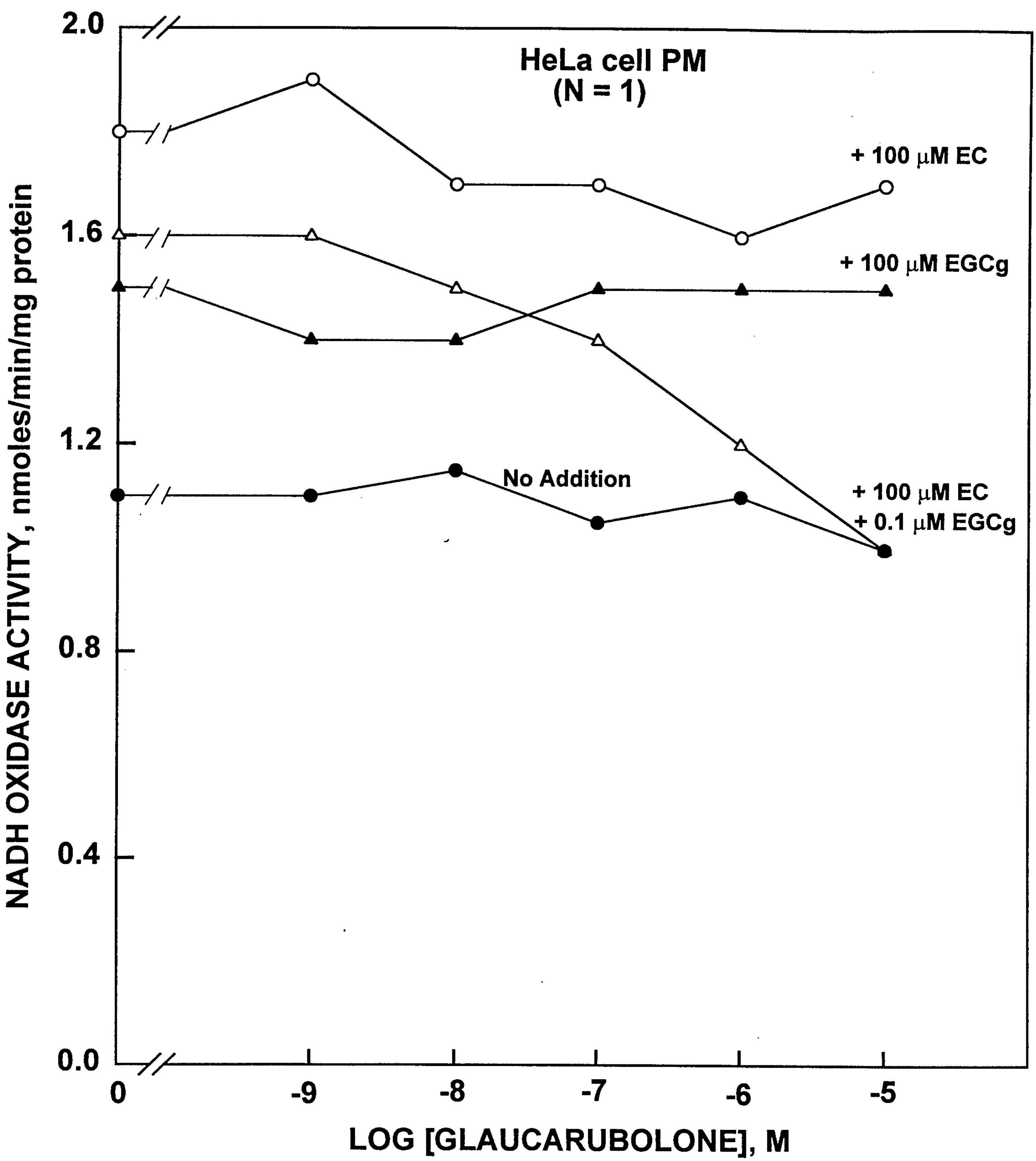

HeLa cell PM
(N = 1)
+ 100 µM EC
+ 100 µM EGCg
No Addition
+ 100 µM EC
+ 0.1 µM EGCg
NADH OXIDASE ACTIVITY, nmoles/min/mg protein
2.0
1.6
1.2
0.8
0.4
0.0
0
-9
-8
-7
-6
-5
LOG [GLAUCARUBOLONE], M

Fig. 5

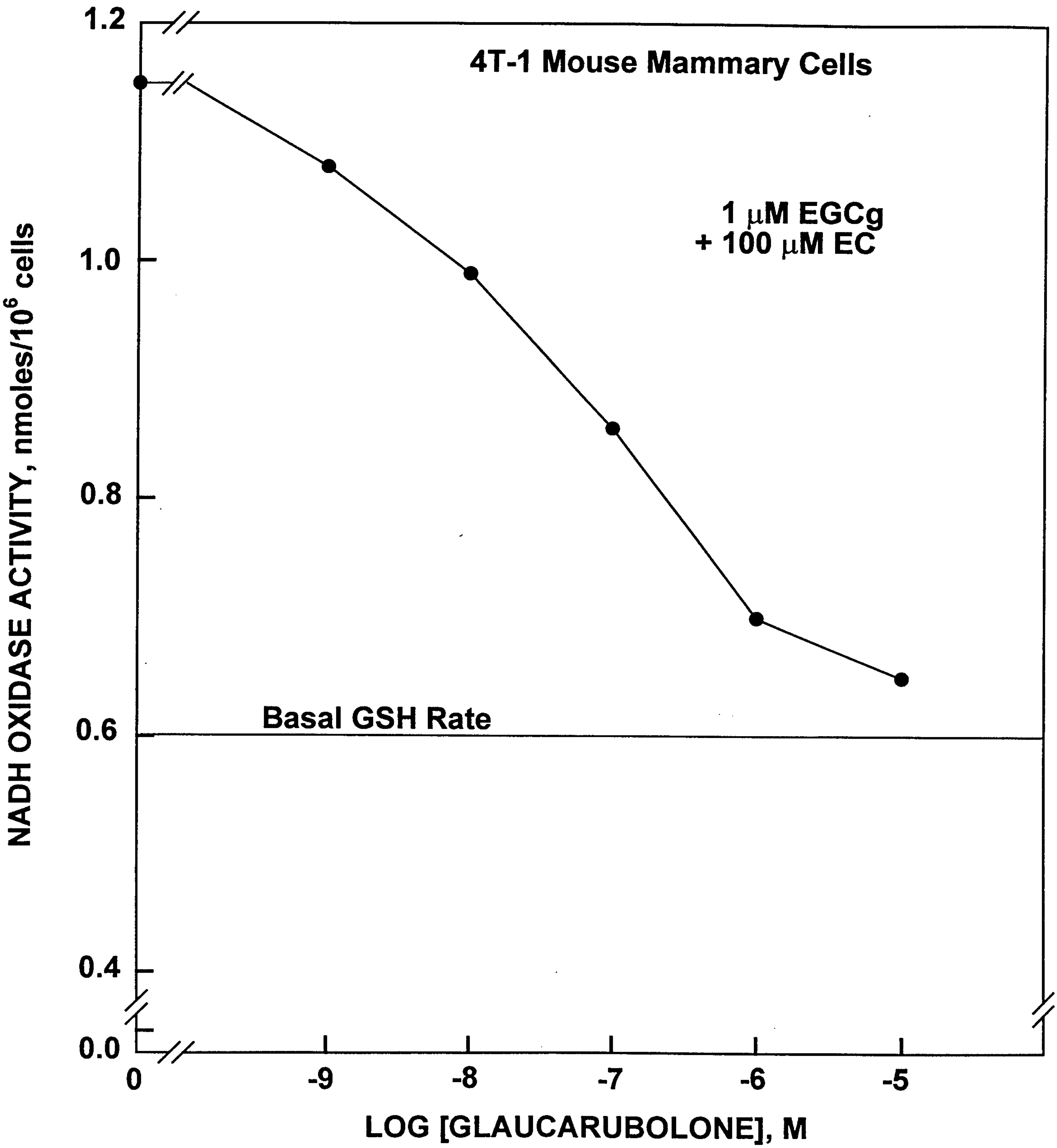

4T-1 Mouse Mammary Cells
1 μM EGCg
+ 100 μM EC
Basal GSH Rate
NADH OXIDASE ACTIVITY, nmoles/10^6 cells
1.2
1.0
0.8
0.6
0.4
0.0
0
-9
-8
-7
-6
-5
LOG [GLAUCARUBOLONE], M

Fig. 6